

**associated with peripheral neuropathy and severe
hyperlactataemia or lactic acidosis in HIV-infected
adults exposed to nucleoside analogues reverse
transcriptase inhibitors**

by

Alejandro Rafael Arenas Pinto

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Department of Primary Care and Population Sciences

Royal Free and University College Medical School

University College London

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Abstract

Studies on mitochondrial dysfunction in HIV-infected adults exposed to anti-retroviral therapy

A significant proportion of HIV-infected patients who require anti-retroviral therapy are or have been exposed to nucleoside analogue reverse transcriptase inhibitors (NRTIs). It has been consistently suggested that most of the NRTI-attributed adverse drug reactions (ADR) are due to mitochondrial dysfunction.

In a sub-analysis of a large randomised clinical trial (Delta) the incidence of peripheral neuropathy (PN) was constant over time in all study arms, which does not support the hypothesis of cumulative toxicity previously proposed for NRTI-induced ADR. Patients taking zidovudine (AZT)/zalcitabine (ddC) combination were more likely to develop PN than patients on AZT monotherapy (RH= 2.30; 95%CI= 1.62 – 3.28). The incidence of PN among patients exposed to zidovudine/didanosine (AZT/ddI) combination was not different from that observed in patients on AZT.

In a multi-centre case-control study including 110 cases of lactic acidosis (LA) or severe hyperlactataemia (HL) patients with < 200 CD4 cell/ μ l were more likely to develop HL/LA than patients with higher levels of CD4 cells (OR=3.44; 95%CI= 1.64 – 7.22). Female patients were found to be at higher risk for HL/LA than men (OR= 4.75; 95%CI= 1.96 – 11.53). Patients exposed to either d4T, ddI or the combination of these two were four to six times more likely to develop HL/LA than patients taking other NRTIs based combinations. Interestingly, cases of HL/LA were exposed to d4T for shorter periods of time than controls.

Almost 10 % of the cases included in the study were asymptomatic at the time of diagnosis. All these symptom-free cases had blood lactate ranging between 5 and 7 mmol/l. Therefore, case definitions for HL or LA based on clinical presentation may underestimate the magnitude of the problem.

Declaration

Under the appropriate supervision, I developed the design for all three studies included in this thesis: the sub-analysis of Delta trial on peripheral neuropathy (chapter 3), the systematic review on lactic acidosis (chapter 4) and the multi-national case-control study on risk factors for severe hyperlactataemia and lactic acidosis (chapters 6 and 7).

In addition, the data collection for the systematic review and the case-control study were also performed by me. However, as described in chapter 5, for the case-control study in those centres where language or legal barriers prevented me to have direct access to the participant patients' notes, study proformas were completed by local researchers and audited in situ by me. Study proformas were completed by Shula Grivell, Erika Erig, Esteban Martinez, Signe Westring Worm, Sophie Herbert, Mooka Busi, Turner Overton and Joanna Turner at the Academic Medical Center, Amsterdam, University Hospital, Zurich, Hospital Clinic, Barcelona, Hvidovre Hospital and Righospitalet, Copenhagen, Alfred Hospital, Melbourne, St Jame's Hospital, Dublin, Washington University, St Louis and North Manchester General Hospital, Manchester respectively.

The statistical analyses of the data collected in both the systematic review (chapter 4) and the case-control study (chapter 6) were done by me. The analysis of the data extracted from the Delta trial database was performed in collaboration with Krishnan Bhaskaran from the Clinical Trials Unit MRC.

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Papers and abstracts arising from this work

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List of abbreviations

3TC	Lamivudine
ABC	Abacavir
ADP	Adenosine di-phosphate
ADR	Adverse drug reaction
AIDS	Acquired immune deficiency syndrome
ART	Anti-retroviral therapy
ATP	Adenosine tri-phosphate
AZT	Zidovudine
CI	Confidence interval
d4T	Stavudine
ddC	Zalcitabine
ddl	Didanosine
DNA	Deoxyribonucleic acid
FADH ₂	Flavin adenine dinucleotide
HAART	Highly active anti-retroviral therapy
HBV	Hepatitis B virus
HCV	Hepatitis C virus
HIV	Human immune deficiency virus
HL	Hyperlactataemia
HU	Hydroxyurea
IQR	Inter-quartile range
LA	Lactic acidosis
MRC	Mitochondrial respiratory chain
mtDNA	Mitochondrial DNA
NADH	nicotinamide adenine dinucleotide
nDNA	Nuclear DNA
NNRTIs	Non-nucleoside reverse transcriptase inhibitors
NRTIs	Nucleoside analogue reverse transcriptase inhibitors
OR	Odds ratio

PBMC	Peripheral blood mononuclear cells
PI	Protease inhibitors
PN	Peripheral neuropathy
RH	Relative Hazard
ROS	Reactive oxygen species
SD	Standard deviation
TDF	Tenofovir
WHO	World Health Organisation

CHAPTER 1

Introduction

The aim of this thesis was to study clinical and epidemiological factors associated with nucleoside analogue reverse transcriptase inhibitors (NRTIs) associated adverse drug reactions (ADR) in HIV-infected patients. Particularly to identify risk factors for such toxic effects using two models: peripheral neuropathy (PN) and lactic acidosis – severe hyperlactataemia (LA/HL).

Most of the long-term or serious NRTI-associated ADR have been proposed to be due to mitochondrial dysfunction induced by these drugs. Evidence supporting the hypothesis of mitochondrial impairment as the explanation for NRTI-induced ADR has been presented. However, the exact mechanism for such mitochondrial harm is still controversial.

Chapter 2 offers background information relevant for the thesis content including basic information on mitochondrial structure and function as well as medical conditions associated with mitochondrial impairment. In addition, background information on NRTIs structure and pharmacology is presented in light of potential mechanisms of toxic effects. Finally, the effect of HIV itself on mitochondrial function is briefly discussed.

It has been proposed that long-term NRTI-associated ADR depend on a cumulative dose of the drug. In chapter 3 the question of cumulative toxicity of NRTIs is assessed using PN as a model for NRTI-induced mitochondrial dysfunction. A brief introduction on NRTI-associated and HIV-induced PN is presented as well as previous research suggesting a cumulative toxicity of NRTIs. The analysis presented was performed using data previously collected for a large randomised clinical trial and therefore the clinical trial is briefly described. The incidence of PN and its association with specific drugs is also analysed.

Lactic acidosis has been proposed as a “proof of concept” for NRTI-induced mitochondrial dysfunction. Chapter 4 describes a systematic review on published cases of LA with the objective of identifying potential risk factors for such a complication. The implications of the findings are analysed in the context of further research needs.

A multi-national case-control study on LA and HL was planned and performed and is described in chapters 5 and 6. Justification for the study and its design are presented and discussed in light of a pilot study performed in advance. Results of this multi-centre study are presented including a descriptive analysis of the cases together with identification for risk factors associated with the study outcome and with mortality. Descriptive analysis of the data collected on cases is presented. This is the largest case-series compiled on HL/LA to date.

Chapter 7 discusses the results presented in chapter 6 analysing not only risk factors for the combined outcome (i.e. HL/LA) but also comparing factors associated with each outcome considered individually. Risk factors for mortality are also analysed.

In chapter 8 a conclusive discussion is presented where the role of dideoxynucleosides in PN and HL/LA is analysed in light of the results presented in chapters 3 and 6. The concept of a special susceptibility for NRTI-induced mitochondrial dysfunction is discussed.

Finally, chapter 8 includes a short discussion on implications and possible consequences of safety issues related to anti-retroviral therapy (ART) on HIV/AIDS control programs, particularly in the developing world. Recommendations for further research are also presented.

CHAPTER 2

Background to the Project

Mitochondria are critically important intra-cellular organelles which perform a variety of biological functions. They are also very peculiar not only because of their unique origin and genetics but also because they impact on almost every cellular function. Mitochondria are present in all nucleated human cells and therefore any impairment in function may generate a significant variety of clinical conditions depending on the organ or tissue affected.

Over the last few decades the importance of mitochondrial biology on human medicine has been increasingly recognised and a significant and still growing list of diseases has been attributed to mitochondrial dysfunctions of different aetiologies (DiMauro & Schon 2003; White 2001). In that context, mitochondrial dysfunctions have been suggested as the common pathway of several proposed adverse drug reactions (ADR) attributed to antiretroviral agents used to treat HIV infection.

2.1 Adverse effects associated with anti-retroviral therapy for HIV infection

The introduction of highly active anti-retroviral therapy (HAART) as standard of care for HIV-infected patients has transformed HIV disease to a chronic condition inducing a significant reduction in both AIDS-related morbidity and mortality (Palella, Jr. *et al.* 1998). Long-term remission of HIV replication is now an achievable goal. Unfortunately, currently available therapeutic options are not capable of eradicating the virus and therefore patients must receive anti-retroviral therapy (ART) for long periods, probably life long, to achieve the goals of HAART.

The last assertion means that patients are now exposed to both therapeutic and toxic effects of ART drugs for long periods of time. Furthermore, because

a combination of ART drugs is the standard of care, patients are now exposed to toxic effects of several drug classes at the same time.

2.1.1 Toxicity as a limiting factor for ART success

The long-term success of HAART has been reported to be challenged by several factors such as virological or immunological failure and toxicity. Observational studies have shown that about 20 – 25 % of those patients who start ART discontinue therapy because of either treatment failure or toxicity during the first eight months of exposure to their initial regimen (Montessori *et al.* 2004).

Interestingly, most of the patients stopping or modifying ART regimens do so because of toxicity. Among 862 treatment naïve patients who started ART, 21 % had to stop therapy because of toxicity whereas the proportion of people doing so because of either virological or immunological failure was 5 % over a median follow-up period of 45 weeks (d'Arminio *et al.* 2000). Similarly, an analysis of a cohort including 556 patients has shown that 64 % of those patients who modified their first ART regimen did so because of patients' choice, poor compliance or toxicity (Mocroft *et al.* 2001).

Furthermore, full adherence has been shown as crucially important to reduce the risk of virological failure (Yeni *et al.* 2004). Therefore, any factor associated with poor adherence is actively favouring the emergence of HIV resistant variations. Importantly, ART-related adverse effects have been shown as a major cause of intentional non-adherence to ART in several studies (Heath *et al.* 2002). Therefore, ART-associated adverse effects may have a negative impact on HAART long-term benefits in at least two different ways, directly causing necessary treatment interruptions or indirectly, facilitating sub-optimal adherence to ART and subsequently promoting viral resistance.

Currently licensed ART drugs include four major classes: a) nucleoside analogues reverse transcriptase inhibitors (NRTIs), which might include nucleotide analogues as well, b) non-nucleoside reverse transcriptase inhibitors (NNRTIs), c) protease inhibitors (PI) and d) fusion inhibitors. Virtually all ART drugs can induce adverse effects, some of which are class effects, and therefore have been described in patients exposed to all or almost all of available drugs from a specific class. On the other hand, some adverse effects are drug specific.

Two or more NRTIs are often used as backbone of many HAART combinations and therefore an important proportion of HIV-infected patients world-wide are or have been exposed to NRTI-associated adverse effects. Most of NRTI-induced long-term adverse or toxic effects have been proposed to be a consequence of mitochondrial dysfunction.

As NRTIs is the drug class most frequently used to treat HIV disease, understanding the nature of the proposed NRTI-induced mitochondrial toxicity as well as the risk factors for specific NRTI-associated adverse effects are critically important for more appropriate and safer management of HIV infection.

2.2 Mitochondria

Mitochondria are the legacy eukaryotic cells received a billion years ago, when aerobic bacteria colonised their primitive ancestors. Primitive eukaryotic cells were unable to use oxygen metabolically, so the parasites offered to their host a more efficient way to produce energy and eventually evolved into mitochondria (Finsterer 2004). Mitochondria are present in all human nucleated cells, so erythrocytes are the only cell type which does not have mitochondria.

Energy production in the form of adenosine triphosphate (ATP) is probably the most relevant of the functions mitochondria perform. However, this organelle

is involved in many other biological processes including pyruvate oxidation, the Krebs cycle, the metabolism of amino acids, fatty acids and steroids and furthermore, mitochondria are likely to be implicated in the process of apoptosis (Newmeyer & Ferguson-Miller 2003).

2.2.1 Mitochondrial morphology and function

It has been observed that there are several hundred or even a thousand mitochondria in each eukaryotic cell (Fig. 2.1). These organelles increase the cell capacity of energy production significantly. The process of anaerobic glycolysis, which transforms glucose to pyruvate in the cytoplasm, is completed in the mitochondria, where pyruvate is oxidised by O_2 to produce CO_2 and H_2O . This mitochondrial aerobic glycolysis produces 15 times more adenosine tri-phosphate (ATP) than the anaerobic cytoplasmic process (Fig 2.2).

Fig. 2.1 Mitochondrial morphology

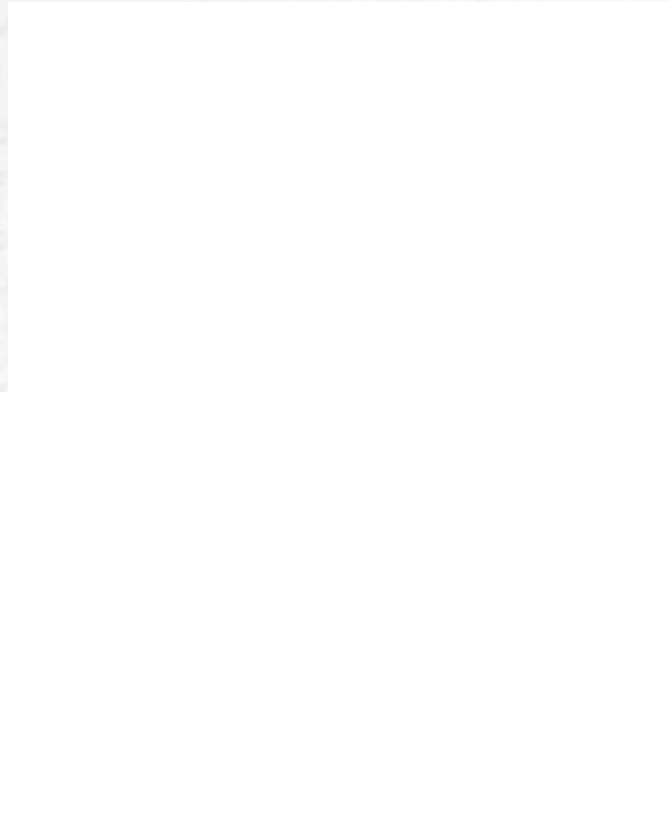


Source: Modified from <http://www.science-art.com/image.asp?id=2205&m=24>

Mitochondria are mobile and plastic structures, which are constantly moving in the cytoplasm and changing their shape. Mitochondria are formed by two membranes, which define two different internal compartments. Between the outer and the inner membranes is the inter-membrane space. As explained

later in this chapter, most of the mitochondrial proteins are assembled in the cytoplasm and imported by the mitochondria. Consequently, there are many copies of transport systems in the outer membrane and the inter-membrane space is occupied by a wide variety of proteins.

Fig. 2.2 Aerobic and anaerobic glycolysis



Modified from End Rev. 2004; 25(5): 807-30

By contrast, the matrix, which is the compartment defined by the inner membrane, has a more selected content. The inner membrane is highly specialised, having a structure which makes it "impermeable" to ions and contains more specialised transport systems than the outer membrane. These two elements, the inner membrane and the matrix, are the metabolically active components of the mitochondrion.

As mentioned before, glucose is transformed to pyruvate in the cytoplasm and can be subsequently transported into the mitochondria. Once in the mitochondrial matrix, pyruvate is transformed to Acetyl-CoA in a process

catalysed by the pyruvate dehydrogenase enzymatic complex. In addition to that, Acetyl-CoA might also be synthesised "*de novo*" as a product of the fatty acid β -oxidation process that is also performed in the mitochondrial matrix. Long-chain fatty acids enter the mitochondria in a process facilitated by carnitine.

Acetyl-CoA is incorporated in the tricarboxylic acid cycle (also known as the citric acid or Krebs cycle) which generates reduced nicotinamide adenine dinucleotide (NADH) and reduced flavin adenine dinucleotide (FADH₂). In addition, FADH₂ is also a product of the fatty acid β -oxidation process. These two molecules (i.e. NADH and FADH₂) are carriers of high-energy electrons, which are transferred to the respiratory chain located in the inner membrane.

The respiratory chain is a highly specialised system of five multimeric protein complexes that have the function of generating ATP. NADH donates high-energy electrons to complex I (NADH-ubiquinone-oxidoreductase or NADH dehydrogenase) whereas FADH₂ does so to complex II (succinate-ubiquinone reductase or succinate dehydrogenase). The process of oxidative phosphorylation implies that electrons transferred to the respiratory chain eventually are combined with molecular oxygen (O₂) to form water. The transport of electrons through the respiratory chain complexes also generates an electrochemical proton gradient across the inner membrane. This gradient is a consequence of the active pumping of H⁺ to the inter-membrane space. (Fig 2.3)

The final step in the oxidative phosphorylation process is the synthesis of ATP. The gradient of protons generated across the inner membrane as a consequence of electron transport through the respiratory chain drives H⁺ back into the matrix. The protons can cross the inner membrane using a hydrophilic channel formed by a membrane-bounded enzymatic complex. This multimeric protein is named ATP synthase because as protons pass through its channel, adenosine diphosphate (ADP) is phosphorylated to ATP using inorganic phosphate.

2.2.2 Mitochondrial respiratory chain

The mitochondrial respiratory chain, as mentioned before, is an electron transport system located in the inner mitochondrial membrane. The chain is formed by more than 80 peptides distributed in five different complexes. Complexes I, III and IV are proton pumps which generate the gradient of protons used by ATP synthase (often called complex V) to generate ATP.

Fig. 2.3 Mitochondrial respiratory chain

Modified from: Diabetes, 2005; 54: 1619

Complex I is the largest component of the respiratory chain and is the acceptor of high-energy electrons from NADH produced by the Krebs cycle. The other entry point to the respiratory chain is complex II, which is the smallest component of the chain and accomplishes the oxidation of FADH_2 also derived from the Krebs cycle. Complex III generates cytochrome c, which is used for the reduction of O_2 to two molecules of H_2O in a reaction catalysed by cytochrome c oxidase (complex IV).

The reduction of O_2 by cytochrome c oxidase, NADH dehydrogenase and some other enzymes involved in the process may be considered as very efficient. Nevertheless, about 12 % of the oxygen consumed during the respiration process is not fully reduced to water but to superoxide anion ($O_2^{\cdot-}$), hydrogen peroxide (H_2O_2) and some other moieties. These reactive oxygen intermediates are usually named reactive oxygen species (ROS) (Di Donato 2000).

ROS can induce oxidative damage to any component of the respiratory chain and therefore induce impairment in the oxidative phosphorylation process. However, in physiological conditions several enzymatic proteins can protect cellular structures from oxidative damage. These enzymes include superoxide dismutase, catalase and glutathione peroxidase which can catalyse the transformation of H_2O_2 into water and molecular oxygen. In addition, antioxidant vitamins E and C can help in defending cells from oxidative damage (Droge 2002).

2.2.3 Mitochondrial genetics

As explained before, mitochondria may have been the result of the evolution of previously independent bacteria. Mitochondria contain several copies of circular double stranded DNA molecules: the mitochondrial DNA (mtDNA). This mtDNA is the sole extra-nuclear DNA in human cells. In addition to that, mitochondria also have their own systems for transcription, translation and protein-assembly (Finsterer 2004).

Human mtDNA has 37 genes and is formed by 16,569 base pairs (Fig. 2.4). Only 13 of the mtDNA genes encode for components of the respiratory chain enzymatic complexes. Seven sub-units of complex I (NADH dehydrogenase), one sub-unit of complex III (ubiquinone c reductase), three sub-units of complex IV (cytochrome oxidase) and two sub-units of complex V (ATP synthase) are encoded by mtDNA genes. Interestingly, none of the five complexes of the respiratory chain is fully encoded by mtDNA, whereas

nuclear DNA (nDNA) encodes completely the complex II (succinate dehydrogenase). In fact, mtDNA encoded products represent a significant minority of the mitochondrial proteins. Furthermore, not only structural proteins of both mitochondrial membrane and respiratory chain complexes but also those peptides responsible for the tricarboxylic acid cycle and even more critical proteins involved in mitochondrial replication are encoded by nDNA. Nevertheless, peptides encoded by mtDNA are crucially important for mitochondrial function because they represent functional sub-units of the respiratory chain complexes (Stacpoole 1997).

Fig. 2.4 Mitochondrial DNA map



Because of several peculiar characteristics of the mitochondrial dynamic, mtDNA is more prone to suffer mutations than nDNA (see below). Some but not all of the thousands molecules of mtDNA coexisting in a single cell might be pathogenic mutants. Furthermore, different variants of mtDNA molecules may be present in a single mitochondrion. This characteristic of the mitochondrial genetic is called heteroplasmy (DiMauro & Schon 2003; White 2001).

The coexistence of normal and abnormal mtDNA copies in a single cell has an impact in the function of mitochondria. When the normal population of mtDNA outnumbers the amount of variant or mutant copies, the cell is capable of showing normal function. As the proportion of mutant mtDNA copies increases, the cell is likely to express some degree of mitochondrial dysfunction. However, a threshold effect has been described: cells containing mtDNA mutant copies develop mitochondrial dysfunction when the concentration of these abnormal copies is higher than a certain level. That critical level might vary depending on the affected tissue, but it is generally accepted that a single cell requires a high population of aberrant mtDNA copies to develop functional impairment. For inherited mitochondrial diseases it has been estimated that the proportion of mutant mtDNA molecules might be around 70 - 80 % of the total mtDNA content to affect the normal function of the affected cell (Stacpoole 1997). Nevertheless, it is important to highlight that the threshold for cellular dysfunction can be considerably lower in tissues which are highly dependent on oxidative phosphorylation such as brain, heart, skeletal muscle, retina, renal tubules and endocrine glands (DiMauro & Schon 2003).

Because of the replicative or mitotic segregation, the proportion of aberrant variants of mtDNA might differ after cell replication. The distribution of mitochondria in the cell before starting the mitotic process is probably almost at random. As a consequence of that, the proportion of aberrant mtDNA present in the daughter cells can be different from the proportion noted in the mother cell and between the daughter cells. This peculiar characteristic of the

mitochondrial genome might explain the unequal distribution of aberrant mtDNA copies in a given tissue and even more, the heterogeneity of mitochondrial function in this given tissue (White 2001).

Mitochondrial DNA is believed to be almost exclusively maternally inherited. The content of mitochondria in sperm cells is relatively small in comparison with the amount of mitochondria in the ovum. Even more, the segment of the spermatozoa that contains most of the mitochondria apparently is lost during fertilisation (Stacpoole 1997). In consequence, virtually all mitochondria in the zygote are from maternal origin. Nonetheless, a case of paternally inherited mitochondrial myopathy has recently been published. Schwartz and Vissing determined that 90 % of this patient's muscle mtDNA had the mutation responsible for the myopathy. According to the authors, this mutation was paternally inherited (Schwartz & Vissing 2002).

2.2.4 Polymerase gamma

In eukaryotic cells the process of DNA replication is catalysed by a family of enzymes called DNA polymerases. There are five DNA polymerases that have been described in mammalian cells. DNA polymerases α , β , δ and ϵ are responsible not only for DNA replication but also for repairing DNA strands. All these four DNA polymerases are located in the cellular nucleus.

The other DNA polymerase that has been identified in mammalian cells is located in mitochondria and is called DNA polymerase γ . This polymerase γ is the sole enzyme responsible for mtDNA replication. In addition, some studies have suggested that polymerase γ may also be involved in mtDNA repair (Lecrenier & Foury 2000). Although some DNA repair capacity has been demonstrated in the mitochondria, it has been proposed that this capacity is limited and much less efficient than the capacity observed in the nucleus.

DNA polymerase γ contains two sub-units. The largest sub-unit shows both polymerase and exonuclease activities whereas the smallest or accessory unit provides strong and tight binding to the DNA template, allowing high processivity¹ in mtDNA synthesis. It has therefore been proposed that mtDNA deletions might be replicated more efficiently than the normal variant. The proposed preferential replication of abnormal mtDNA copies might accelerate the accumulation of mutant mtDNA copies and therefore, the achievement of the necessary threshold for cellular dysfunction (Lewis *et al.* 2001).

High rate of mtDNA replication has been demonstrated not only during cellular division but also during electrical stimulation of muscular cells and normal physical muscular systematic training (Lecrenier & Foury 2000). Similarly, it has been demonstrated that oxidative stress induced by H₂O₂ promotes an increase in both mitochondrial mass and mtDNA (Lee *et al.* 2000). However, DNA polymerase γ must be encoded by nDNA as this enzyme can be expressed in the absence of mtDNA (Davis *et al.* 1996). That suggests that mitochondrial function is regulated at nuclear level as both synthesis of critical functional and structural mitochondrial proteins and mtDNA replication are controlled by nDNA (Shoubridge 2001).

There are several factors that make mtDNA more prone to suffer mutations. DNA polymerase γ has been proposed to have limited capacity to repair mismatching errors during the replication process (Feng *et al.* 2001). In addition, the mtDNA replication rate is much greater than the rate of nDNA replication. Furthermore, mtDNA is constantly exposed to factors capable of inducing oxidative damage on its structure such as ROS (Lecrenier & Foury 2000). In addition to that, mtDNA is not protected by histones. Finally, in addition to its special susceptibility to mutation, mtDNA is much more likely than nDNA to express each mutation. Almost all mtDNA genes are translated whereas nDNA has many silent genes.

¹ Processivity: in DNA replication when the replication complex assembles at the origin (s) of replication and then performs template-directed synthesis of DNA over virtually the entire genome without dissociation (Reddy MK *et al.* J Biol Chem 1992, 267 (20): 14157 - 166).

2.3 Mitochondrial dysfunction associated syndromes

Mitochondrial dysfunction has been associated with a variety of clinical syndromes. The most frequent causes of mitochondrial dysfunction in the general population are inherited disorders from both nDNA and mtDNA. Mutations directly affecting the respiratory chain are classified as primary genetic disorders whereas those conditions affecting nuclear encoded proteins are considered secondary defects of the respiratory chain.

2.3.1 Inherited mitochondrial syndromes

About one hundred mtDNA mutations have been associated with human disease (Schapira 2002). These primary defects of the mitochondrial respiratory chain have been presented as rare conditions. However, as their estimated prevalence might be in the range of 10 to 15 cases per 100,000 people (DiMauro & Schon 2003), one could assume that they may be considered as uncommon rather than rare. Primary mitochondrial diseases are usually diagnosed during infancy, but late onset expression of disease has been reported (Schapira 2002).

All mitochondrial diseases described to date (Table 2.1) share some important characteristics, the most important being clinical heterogeneity. This group of disorders can affect a single organ or can induce systemic disease. Furthermore, there is no clear relationship between the site or type of mutation and its clinical expression: A given mutation might cause different clinical presentations while a specific clinical syndrome might be consequence of several different mutations (Leonard & Schapira 2000a). There are however, some important exceptions to this rule. Patients with MERRF (myoclonus epilepsy with ragged-red fibres) and Leber's optic neuropathy have specific mutations always associated with each condition (DiMauro & Schon 2003).

Table 2.1 Inherited mitochondrial diseases: clinical classification

Modified from European Journal of Neurology 2004; 11: 163 - 86

Despite the previously mentioned variability, almost all mtDNA disorders usually develop lactic acidosis (DiMauro & Schon 2003). Furthermore, impairment of the central nervous system is frequently seen and might be the main feature of many mitochondrial syndromes such as MELAS (myopathy, encephalopathy, lactic acidosis, and stroke-like episodes) or MERRF. Leber's hereditary optic neuropathy (LHON) is the most common cause of blindness in healthy young men. It is important to highlight that a significant proportion of asymptomatic people can show mutations in mitochondrial ND genes suggesting that additional factors might contribute to the expression of LHON (DiMauro & Schon 2003; Leonard & Schapira 2000a).

Mitochondrial function may also be affected by defects in processes not directly linked with the respiratory chain. Conditions such as Friederich's ataxia, hereditary spastic paraparesis, Wilson's disease, Parkinson's disease, Huntington's disease and even Alzheimer's disease have all been proposed as showing some degree of impairment in mitochondrial function as a consequence of nuclear genetic defects (Leonard & Schapira 2000b).

2.3.2 Acquired mitochondrial dysfunction

Ischemia can induce mitochondrial dysfunction. However, the most frequent cause of acquired mitochondrial dysfunction is toxicity. Several chemicals can induce mitochondrial impairment by different mechanisms.

Ethanol is probably the most frequent inducer of acquired mitochondrial disease. It has been shown that ethanol can accelerate oxidative damage of mtDNA and can also induce a significant reduction in some critical mitochondrial enzymatic activities affecting the respiratory chain (Fromenty & Pessayre 1997; Sebastian & Setty 1999). Nevertheless, other chemicals have also been shown to be mitochondrial toxins including drugs such as amiodarone, anthracyclines, aspirin, chloramphenicol, hydroxyurea, tetracycline, valproic acid and nucleoside analogues of the HIV reverse transcriptase (Moyle 2000).

Although a direct effect on mitochondrial function and/or structure has been demonstrated in in-vitro studies with most of the drugs associated with mitochondrial dysfunction, it has also been suggested that at least some of them might be mild toxins. It has been proposed therefore that these drugs are capable of unmasking sub-clinical inherited mitochondrial dysfunction (Moyle 2005a; Cherry & Wesselingh 2003).

It has also been proposed that mitochondrial toxins may have an additive effect, so using two or more of these drugs concomitantly might accelerate the development of clinical expression of mitochondrial dysfunction (Moyle 2000).

2.4 Pharmacology of nucleoside reverse transcriptase inhibitors

Nucleoside analogues of the HIV reverse transcriptase (NRTI) were the first drug class to be licensed for use in patients with HIV disease. Zidovudine (AZT) was licensed in the late eighties to treat patients with AIDS in the US and in Europe. Soon after regulatory agencies from the developed world granted their approval for human use to other related compounds. Furthermore, NRTIs are the backbone of most HAART combinations currently in use. Taken together, one can appreciate that a significant proportion of the treated HIV-infected population is or has been exposed in the past to NRTIs and their adverse effects.

2.4.1 Nucleoside analogues

There are seven NRTIs currently approved for the treatment of HIV infection or AIDS: zidovudine (AZT or ZDV), zalcitabine (ddC), didanosine (ddI), stavudine (d4T), lamivudine (3TC), emtricitabine (FTC), and abacavir (ABC). All these compounds are derivatives of the natural nucleosides adenosine, cytosine, guanosine and thymidine. Chemical structures of some of the licensed NRTIs are shown in Fig 2.5.

As structural analogues, NRTIs share with natural nucleosides several properties and therefore they can act as alternative substrates for both HIV reverse transcriptase and human DNA polymerases. It is important to note that not all human DNA polymerases show the same level of affinity to NRTIs.

DNA polymerase γ and possibly DNA polymerase β are the more likely to incorporate NRTIs (Wright & Brown 1990). Nevertheless, NRTIs are poorly incorporated to the DNA forming chain by human polymerases when compared to HIV-reverse transcriptase.

Fig. 2.5 NRTI structure

Source: http://en.wikipedia.org/w/index.php?title=Main_Page&oldid=98546089 (accessed January 30, 2007).

The most important structural difference between NRTIs and natural nucleosides is the 3'-OH substitution of the deoxyribose sugar. This position is responsible for the 3'-5' union with the next nucleotide leading to elongation of the new DNA chain. However, NRTIs show either a lack of the 3'-OH substitution or a modification of that substitution (AZT) which prevents the 3'-5' bond formation. Therefore NRTIs can induce transcription impairment by at least two different mechanisms 1) competition with natural nucleic acids to be incorporated in the forming DNA chain and 2) premature termination of DNA chain elongation (Kakuda 2000; Anderson *et al.* 2004).

2.4.2 Pharmacology of NRTIs

Pharmacologically, NRTIs must be considered to be pro-drugs as they need to be structurally modified to have pharmacological effects. In a similar way to natural nucleosides, NRTIs must be phosphorylated to become substrates for

either the HIV reverse transcriptase or human DNA polymerases. NRTIs phosphorylation is an intracellular process, which requires participation of several enzymes. Nevertheless, the final step in this activation process, the formation of the triphosphorylated moiety, is probably common to all NRTIs and is catalysed by nucleoside diphosphate kinase. Nevertheless, it has been proposed that ABC has a unique phosphorylation pathway, and that might at least partially explain the differences seen between ABC and other NRTIs toxicity profiles. ABC is phosphorylated once before being deaminated (to form carbovir-MP) in the cytosol and then is phosphorylated twice to form the active moiety (carbovir-TP) (Kakuda 2000).

Intracellular NRTIs phosphorylation has been described as a process dependent on the cellular status. In vitro studies have shown that non-activated or resting cells might phosphorylate more effectively 3TC, ddC and ddI, whereas activated cells are more efficient in phosphorylating d4T and AZT (Kakuda 2000). However, studies addressed to compare NRTIs phosphorylation in resting and activated cells have shown that the actual intracellular concentration of ddC, ddI and 3TC triphosphate may be up to 2 – 4 fold higher in active cells when compared with resting cells (Anderson *et al.* 2004). In that context, the effectiveness of NRTIs might depend on cellular activation as probably also does toxicity if it is associated with concentration of active triphosphate moieties in the intracellular compartment.

Chronic systemic cell activation has been described in patients with HIV disease, showing a direct association between the level of cell activation, measured as concentration of pro-inflammatory markers, and the severity of the disease (Hestdal *et al.* 1997). Similarly, several studies have proposed that severely ill HIV-infected patients have a higher risk of developing NRTI-associated adverse events (Joly *et al.* 2002). Recent clinical studies have shown that the intracellular concentration of triphosphorylated AZT and 3TC seems to be higher in those individuals with lower CD4 cell counts and also in those with a higher baseline HIV viral load (Anderson *et al.* 2003). In addition, similar results were found in female patients when compared with males.

Finally, Anderson et al. also found that both baseline HIV viral load and female sex were the only variables independently associated with time to reach less than 50 HIV copies/mm³ after starting ART (Anderson *et al.* 2003). It does seem that cell activation and female gender may be associated with an increase in the anti-viral activity of NRTIs. These two variables have also been proposed as risk factors for NRTI-associated adverse events (Currier *et al.* 2000), suggesting therefore that triphosphorylated NRTIs are responsible for both therapeutic and toxic effects.

2.5 NRTI-associated adverse effects

During pre-licensing clinical trials on NRTIs, several adverse effects were identified. Some of them have been described as mild and usually self-limited. Examples of these are headache, insomnia and gastrointestinal symptoms (nausea, vomiting and diarrhoea) which have been associated with AZT, ddC, d4T and even ABC. Nevertheless, serious adverse effects such as peripheral neuropathy or anaemia were considered as dose-limiting toxicities in clinical trials using AZT, ddC, d4T and ddI.

The long-term adverse events associated with NRTIs show a remarkable similarity to the clinical manifestations of mitochondrial diseases (Finsterer 2004). Furthermore, several studies have demonstrated the presence of abnormal mitochondria and/or mtDNA impairment in HIV-infected patients who develop complications such as peripheral neuropathy or bone marrow suppression. Other proposed NRTI-associated adverse events, such as pancreatitis, lactic acidosis and even peripheral lipoatrophy might also be attributable to NRTI-induced mitochondrial dysfunction.

2.5.1 The Polymerase gamma hypothesis

Since NRTIs are structurally very similar to natural nucleosides, the potential for them inhibiting human DNA polymerases exist. However, as was

mentioned before, in-vitro studies have shown that nuclear polymerases are less likely to incorporate NRTIs than the HIV reverse transcriptase. Nevertheless, mitochondrial DNA polymerase γ has been shown to be particularly susceptible to incorporate NRTIs. Because human polymerase γ is the sole enzyme responsible for replication and repair of mtDNA, it is logical to assume that NRTIs can lead to mtDNA depletion causing eventually mitochondrial dysfunction (Brinkman & Kakuda 2000; White 2001).

Several in vitro studies have shown that NRTIs can in fact induce mtDNA depletion. Studies using human lymphoblastic cells (Molt-4 and CEM) have shown that it is possible to induce mtDNA depletion in cultured cells by exposure to NRTIs (Chen *et al.* 1991; Martin *et al.* 1994). Nevertheless, it has also been highlighted that not all NRTIs have the same capacity of inducing mtDNA depletion. In vitro studies have shown ddC as the strongest inducer of mtDNA depletion among NRTIs licensed to treat HIV disease. Similarly, almost all studies presented to date have noticed that ABC and 3TC are the weakest inducers of mtDNA depletion (Birkus *et al.* 2002; Martin *et al.* 1994; Chen *et al.* 1991). Other NRTIs such as ddI, d4T and AZT have been allocated in different points in the scale by different authors. Nevertheless, most of the studies suggest that AZT is a weaker inducer of mtDNA depletion when compared with either ddI or d4T. Tenofovir (TDF), a nucleotide reverse transcriptase inhibitor, has been shown to be at least as weaker inducer of mtDNA depletion as ABC or 3TC (Birkus *et al.* 2002).

As early as 1991, Arnaudo *et al.* reported a significant reduction in the content of mtDNA in muscle biopsies from nine patients with AZT-induced myopathy when compared with AZT naïve patients (Arnaudo *et al.* 1991). More recently, Masanés *et al.* found significant mtDNA depletion (52 %, 74 %, and 56 % respectively) in three patients with AZT-induced myopathy when compared with controls (Masanes *et al.* 1998).

MtDNA content has also shown to be reduced in different cell types from patients exposed to different NRTIs. Patients co-infected with hepatitis C virus

(HCV) and HIV have been shown as having a significant reduction of the mtDNA : nDNA ratio in hepatocytes only when they are exposed to either d4T, ddI or ddC as part of their HAART combination. No difference was found in the content of mtDNA in those patients receiving “d drugs” sparing regimens when compared with controls (HCV infected patients but HIV negative) (Walker *et al.* 2004). Severe episodes of hyperlactataemia and pancreatitis have been described in HIV-HCV co-infected patients on regular dideoxynucleoside-based ART when exposed to anti-Hep C medication (i.e. ribavirin). It has been suggested that those patients on ddI-based combinations are at higher risk of developing clinically evident mitochondrial dysfunction when exposed to ribavirin (Bani-Sadr *et al.* 2005). Interestingly the last study failed to demonstrate any increased risk for mitochondrial toxicities in patients receiving d4T without concomitant exposure to ddI.

Compared with HIV-infected but ART-naïve patients, individuals receiving both AZT and d4T based regimens have been shown to have mtDNA depletion in adipocytes. By contrast, those individuals treated with non-thymidine NRTIs (i.e. other than AZT or d4T) have been reported as having similar levels of adipocyte mtDNA compared to ART-naïve controls. Nevertheless, the effect of AZT on adipocyte mtDNA is not the same as the effect induced by d4T on this cell type. In the same study, patients treated with d4T showed more severe mtDNA depletion than AZT treated patients. Furthermore, after switching from d4T to either ABC or even AZT, patients showed a significant recovery in the level of their mtDNA in adipocytes over 1 to 24 months (Hammond *et al.* 2004).

Peripheral blood mononuclear cells (PBMC) have been proposed as a suitable target to assess mitochondrial function in patients exposed to NRTIs (Henry *et al.* 2002; McComsey *et al.* 2002). Comparing patients on AZT/3TC with patients on d4T/3TC or ART-naïve, López *et al.* have reported that those individuals receiving d4T containing combinations showed not only a significant reduction in their mtDNA content but also significant decrease in the activity of respiratory chain complexes III and IV (Lopez *et al.* 2002).

Previously, Côté et al. had presented data showing that HIV-infected patients exposed to NRTIs showed a significant reduction in the mtDNA content of PBMC (from buffy coat) when compared with healthy volunteers. Interestingly, that study also showed a significant recovery in mtDNA content after stopping ART, suggesting reversibility of the toxic effect caused by NRTIs on mitochondria (Cote *et al.* 2002). It is important to mention that all NRTI-exposed patients in Côté's study had symptomatic hyperlactataemia at the time of being tested and all of them were receiving d4T based regimens.

However, it has been proposed that NRTI-induced mitochondrial toxicity may be tissue specific and therefore, evidence of mitochondrial dysfunction such as mtDNA depletion may not be found in every cell type in a given patient. A study designed to quantify mtDNA in subcutaneous adipose tissue from lower limb biopsies in HIV-infected adults has shown that those patients receiving at least one dideoxynucleoside ("d-drug") had significant reduction in the content of mtDNA when compared with patients off treatment. The same study failed to show any association between the level of mtDNA in PBMC and exposure to NRTIs (Cherry *et al.* 2002).

Similarly, a case of fatal lactic acidosis with massive hepatic steatosis in a patient receiving AZT monotherapy was published in the early 90's. The liver histology of that patient showed abnormal mitochondria without viral inclusions. Interestingly, ultra-structural studies of skeletal muscle sample did not show any mitochondrial abnormality (Olano *et al.* 1995).

According to the DNA polymerase γ hypothesis (Lewis & Dalakas 1995), NRTIs can induce impairment of mitochondrial function primarily by inhibiting polymerase γ which eventually leads to mtDNA depletion and energy deprivation to the affected cell. However, some recent evidence suggests that mtDNA depletion might not be the only or even the main mechanism responsible for NRTI-associated mitochondrial dysfunction.

A case of lactic acidosis in a patient exposed to AZT/ddl combination therapy was recently published. The interesting point of this particular case is the absence of any mtDNA depletion in muscle cells but the evidence of a significant impairment of the respiratory chain activity (Miller *et al.* 2003). Similarly, Walker *et al.* have presented in-vitro data suggesting that AZT and AZT/3TC combination in cultured cells can induce a rise in lactate without affecting the actual mtDNA content (Walker *et al.* 2001). Furthermore, a recent study has shown that patients with either peripheral lipoatrophy or peripheral neuropathy had a significant reduction in the mtDNA : nDNA ratio when compared with either HIV-infected patients who did not have any ART-associated ADR or to controls (Rabing *et al.* 2004). Interestingly, in Rabing's study those patients treated with d4T containing combinations had significantly lower mtDNA : nDNA ratio than those patients exposed to AZT based combinations. Similarly, it has recently been shown that patients exposed to either AZT/ddl or AZT/ddC combination therapies had more pronounced mtDNA depletion in PBMC when compared to patients receiving AZT monotherapy after 48 weeks (Reiss *et al.* 2004).

2.5.2 Other possible mechanisms

A few years after introducing the polymerase γ hypothesis, Lewis *et al.* have now proposed a new multi-factorial hypothesis as an explanation for the NRTI-induced mitochondrial dysfunction. According to this new proposition NRTIs may induce three different pathological processes which eventually leads to mitochondrial dysfunction. These three mechanisms are: 1) Energy deprivation due to mtDNA depletion, 2) Mitochondrial oxidative stress, and 3) mtDNA mutations (Lewis *et al.* 2001). Although this integrated hypothesis includes another two mechanisms, the authors still consider mtDNA depletion as the first step in the process.

Mitochondrial sub-units and therefore mtDNA are particularly susceptible to oxidative stress². MtDNA is constantly exposed to the highly oxidative mitochondrial environment. In fact, mitochondrial generation of super-oxide anion radical ($O_2^{\cdot-}$) represents the major intracellular source of oxygen radicals under physiological conditions. Because of that, the steady state concentration of $O_2^{\cdot-}$ in the mitochondrial matrix is between 5 to 10 fold higher than in the cytosol and the nucleus (Cadenas & Davies 2000). In that context, ROS are more likely to induce impairment not only of mtDNA but also mitochondrial structural and functional proteins than to other cellular structures.

Most of the oxygen available for cell respiration or oxidative phosphorylation is reduced in a process catalysed by cytochrome oxidase (Complex IV) to produce water ($O_2 + 4e^- + 4H^+ \longrightarrow 2H_2O$). Cytochrome oxidase is the final electron acceptor in the respiratory chain, so in order to allow electrons to flow through the chain it needs to give up its electrons by reducing O_2 (Cadenas & Davies 2000). Although mitochondria represent a highly efficient system, between 2 – 4 % of the total of electrons transported by the respiratory chain results in the reduction of O_2 to H_2O_2 instead of water under physiological conditions (Lewis *et al.* 2001). Obviously, alterations in the electron flow through the respiratory chain by any cause can increase ROS production substantially.

Several components of complexes I, II and III have the capacity of inducing the generation of $O_2^{\cdot-}$ (Fig. 2.6). In addition, the monoamine oxidase located in the outer mitochondrial membrane catalyses the formation of H_2O_2 , which is mainly responsible for increasing the concentration of ROS within both the mitochondrial matrix and the cytosol. The proximity of mtDNA to sites of ROS production and the lack of histones make it very vulnerable to oxidative damage. In fact, the level of oxidised bases in mtDNA is generally 10 to 20 fold higher than in nDNA (Cadenas & Davies 2000).

² Oxidative Stress: A disturbance in the balance between the production of reactive oxygen species (free radicals) and anti-oxidant defences.
(Betteridge DJ. What is oxidative stress? *Metabolism* 2000; 49 (Suppl 2): 3 – 8)

Fig. 2.6 Respiratory chain showing potential sites of superoxide anion formation

Source: *Lab Invest* 2001; 81(6): 781

It has been proposed that AZT or its metabolites have an inhibitory effect on the mitochondrial oxidative energy production that might not be associated only with mtDNA depletion. It has been demonstrated that short-term exposure of rats to AZT increases ROS production in heart cells, leading also to a significant increase in lipid peroxidation and protein oxidation without any evidence of mtDNA depletion (Szabados *et al.* 1999). Furthermore, the last study found some increase in the amount of mtDNA in heart cells of AZT exposed animals. Similarly, in vitro data using lymphoid cells (U937 and MOLT4) cultured with AZT showed significant inhibition of various mitochondrial enzymes including ADP/ATP translocator, adenylate kinase, NADH-cytochrome c reductase and those of NADH-linked respiration. As consequence of that, a rapid decrease in ATP production has been noted in human cells exposed to AZT potentially leading to overproduction of ROS (Yamaguchi *et al.* 2002). In both, animal models and cultured human cells (lung fibroblasts MRC-5) exposed to AZT it has been noticed that mtDNA increases as consequence of oxidative stress (Lee *et al.* 2000; Szabados *et al.* 1999). This initial rise in mtDNA might be a compensatory mechanism and suggest that mtDNA depletion might not be the first step in the NRTI-induced mitochondrial dysfunction. Similarly, mtDNA content increases with ageing in

normal cells and it has been proposed to be associated with oxidative damage as well.

In addition, oxidative damage caused by ROS may induce single strand DNA breaks, lipid peroxidation and protein oxidation (Szabados *et al.* 1999). These three mechanisms may play significant roles in the development of mitochondrial dysfunction associated with NRTIs exposure. MtDNA deletions may be detected as early as 12 to 15 days after exposure of human lymphoid cells to AZT (Yamaguchi *et al.* 2002). Finally, ROS may also induce DNA base modification. In that context, it has been proposed that those random mtDNA mutations are likely to induce complex I inactivation because a large proportion of its components are mtDNA encoded (Lewis *et al.* 2001). Even more, inhibition of complex I of the respiratory chain may promote an increase in the oxidative stress completing a vicious circle.

In summary, the new hypothesis proposed by Lewis *et al.* suggests that NRTI-induced mitochondrial dysfunction may be consequence of three different and complementary mechanisms. These mechanisms include mtDNA depletion secondary to polymerase γ inhibition that leads to energy deprivation, concomitant mitochondrial oxidative stress which lead to lipid, protein and DNA oxidative damage and finally mtDNA mutation which can be consequence of either oxidative damage, impaired mtDNA replication or transcription (Lewis *et al.* 2003).

Finally, a recent report has shown that short term (two weeks) exposure to either AZT/3TC or d4T/3TC combinations may induce a significant reduction in mitochondrial gene expression (COX1, COX3 and Cyt b) in adipocytes from healthy volunteers (Mallon *et al.* 2004b). The same group has also presented some evidence suggesting that NRTIs can induce inhibition of mtRNA transcription by mechanisms other than mtDNA depletion in both adipocytes and monocytes (Mallon *et al.* 2004a). In addition, they have proposed that the same NRTIs combinations may also induce a decreased nuclear gene expression of some genes implicated in lipid metabolism (PPAR γ) suggesting

the presence of feedback mechanisms between mitochondria and the nucleus which may be relevant in the pathogenesis of lipoatrophy associated with NRTIs (Mallon *et al.* 2005). In summary, one could speculate that NRTIs may induce selective gene expression reduction in both mitochondria and nucleus, which may play a significant role in the pathogenesis of lipoatrophy and possibly other NRTI-associated adverse events, independently of polymerase γ inhibition and irrespective of any effect induced by HIV itself.

In addition, Miró *et al.* have recently reported that asymptomatic HIV-infected patients (N = 17) exposed to d4T/ddI had significant mtDNA depletion compared with controls (ART naïve). Interestingly, despite having significantly reduced mtDNA content in PBMC, patients treated with d4T/ddI showed normal mitochondrial function, suggesting that transcriptional or post-transcriptional mechanisms may compensate, at least initially, in NRTI-induced mtDNA depletion (Miro *et al.* 2004b).

2.5.3 Clinical aspects of NRTI-induced mitochondrial toxicity

As has been mentioned before, there are striking similarities between NRTI-associated adverse events and mitochondrial diseases. In fact, most of the long-term or serious NRTI-associated adverse effects could be explained by mitochondrial dysfunction. Lactic acidosis is the best example.

Bone marrow suppression, myopathy, cardiomyopathy, peripheral neuropathy, pancreatitis, hyperlactataemia and lactic acidosis, hepatic steatosis and peripheral lipoatrophy have all been associated with NRTI-induced mitochondrial dysfunction. The level of evidence supporting the association between mitochondrial dysfunction and each of these complications varies. However, in all cases in vitro and in vivo data suggest that NRTIs may play a significant role in pathogenesis, even when other factors such as HIV infection itself may also be determinant in the clinical expression of them.

Distal symmetrical peripheral neuropathy was identified as the main dose limiting factor during early phase I/II clinical trials of ddC, d4T and ddI. Although NRTI-associated peripheral neuropathy (PN) is clinically indistinguishable from HIV-induced PN, NRTI-related PN has been described as more likely to be painful and to have a more acute course, with an abrupt onset and progressing more rapidly when compared with HIV-induced PN (White 2001). In addition, it has been suggested that high level of blood lactate may be a marker of toxic PN, suggesting that mitochondrial dysfunction is the underlying mechanism (Brew *et al.* 2003).

In vitro studies have shown that nerve cells exhibit mtDNA depletion, alterations in mitochondrial structure and shape and an increase in intracellular lactate levels when they are exposed to ddC and ddI (Keilbaugh *et al.* 1991). Furthermore, in vitro studies using the same cell line (PC-12) have confirmed mtDNA depletion in cells exposed to both ddC and ddI, but not in cells cultured with AZT, 3TC or d4T. Interestingly, AZT and 3TC were found not to be associated with any toxic change in the PC-12 model but d4T was. Authors have suggested that additional mechanisms, other than mtDNA depletion, may be involved in d4T-induced PN (Cui *et al.* 1997).

As has been highlighted above, there is evidence to support an association between mitochondrial dysfunction and PN potentially caused by NRTIs. However, multiple other mechanisms and variables may play significant roles in inducing clinically evident PN in HIV-infected patients. In fact HIV infection itself can be critically important in this regard. Both low CD4 lymphocyte count and high HIV viral load have been identified as risk factors for PN (Moyle & Sadler 1998; Simpson *et al.* 2002). In fact, according to Simpson *et al.* aggressive control of HIV replication may be beneficial to patients in order to control the severity of symptoms. Furthermore, they found that at least for mild cases of PN, the use of “dd drugs” does not necessarily increase the clinical expression of PN.

As with PN, many other NRTI-associated ADR have been proposed to be due to mitochondrial dysfunction but the possibility of other mechanisms playing significant roles in their clinical expression cannot be excluded. However, as serum levels of lactate at rest or after exercise have been used to suggest cellular oxidative impairment, lactic acidosis and hyperlactataemia are conditions which can be considered markers of mitochondrial dysfunction and therefore, are arguably the best model to assess the effects of NRTIs on mitochondrial function.

Episodes of lactic acidosis have been reported among HIV-infected patients exposed to NRTIs since the early 1990's. This complication has been identified in patients receiving monotherapy with NRTIs, mainly AZT or combination of at least two NRTIs. Nevertheless, in vitro and animal models have also supported the concept of NRTIs causing raised levels of lactate as evidence for mitochondrial dysfunction. Cultured human and rodent cells show significant increase in lactate production when exposed to either ddI or ddC (Keilbaugh *et al.* 1997; Tsai *et al.* 1994). Similarly mice and monkeys exposed to either AZT/3TC or d4T have been documented to have a two to eight-fold rise in blood lactate (Dagan *et al.* 2002).

Mitochondrial dysfunction would be expected to lead to an increase in endogenous lactate production. Leclercq *et al.* have demonstrated a marked increase in endogenous lactate production even in patients with a moderate increase in serum lactate (Leclercq *et al.* 2001). However, even extreme elevations of lactate after exercise can be rapidly re-equilibrated as a result of lactate clearance. HIV-infected patients with lipodystrophy exposed to exhaustive exercise, showed a similar pattern of lactate decline during resting time compared to healthy controls (Roge *et al.* 2002). By contrast, a recent study has shown that HIV-infected patients with hyperlactataemia, exposed to sub-maximal exercise, have a reduced lactate clearance capacity. The decline in the lactate levels in patients on ART was considerably slower in patients with raised levels of lactate at baseline compared to patients with normal baseline levels (Bauer *et al.* 2004). One could speculate that hyperlactataemia

and probably lactic acidosis in HIV-infected patients exposed to NRTIs might be a consequence of both an increased lactate production rate and a reduced lactate clearance capacity.

NRTIs have also been demonstrated to induce mitochondrial dysfunction and hyperlactataemia in the absence of HIV infection. Five out of eight HIV-uninfected children were reported as having persistently elevated blood lactate levels after perinatal exposure to AZT in a study published by Blanche *et al.* (Blanche *et al.* 1999). A more recent study has also found significant mtDNA depletion in infants exposed perinatally to AZT/3TC (N = 10) compared with children born to HIV-uninfected women (Divi *et al.* 2004). Similarly, in a prospective study, HIV-uninfected children exposed to AZT as prophylaxis for mother to child transmission showed significant mtDNA depletion at birth and at one and two years of age when compared with children never exposed to AZT (Poirier *et al.* 2003). Nevertheless, authors of that report have highlighted that most of the AZT-exposed HIV-uninfected children are asymptomatic even when they show significant mtDNA depletion.

2.6 HIV and mitochondrial function

HIV-RNA has been identified in the mitochondria of infected cells. In addition, mitochondrial alterations can be found in patients with the acute HIV syndrome and with chronic infection (White 2001). However, information about mtDNA content or the function of respiratory chain enzymatic complexes in HIV-infected patients who have never received ART is scanty. Miura *et al.* have recently reported that treatment naïve HIV-infected patients have a decreased content of mtDNA in PBMC compared with healthy controls. Furthermore, individuals treated with AZT/3TC or d4T/3TC combinations, but not with AZT/ddC combination, showed a significant recovery of the content of mtDNA in PBMC. The study reported by Miura, although involving a small number of patients (N = 46, of which 13 were ART naïve patients) concludes that the cellular content of mtDNA depends on HIV-disease progression as they found a significant positive association between CD4 cell count and

mtDNA content. In addition, the same study showed an inverse association between HIV viral load and mtDNA content in PBMC (Miura *et al.* 2003).

Similarly, a sub-analysis of the Gilead 903 study has shown that ART naïve HIV-infected individuals (N = 227) had significant reduction in their mtDNA content in PBMC when compared with healthy controls (N = 49). In addition, the same study has shown that those patients treated with a TDF/3TC combination (N = 113) had a significant recovery of their mtDNA content after 48 weeks of therapy. The study failed to demonstrate any mtDNA recovery in the group of patients treated with d4T/3TC combination (Gallant *et al.* 2002).

Nevertheless, HIV has also been proposed to impair mitochondrial function independently of any effect on mtDNA content. A recently published study has shown that PBMC from HIV-infected but treatment naïve patients (N = 25) had a significant reduction in the activity of respiratory chain complexes III and IV when compared with healthy volunteers (N = 25). Furthermore, HIV-infected patients also had a significant reduction in the activity of complex II, which is exclusively encoded by nuclear DNA, concomitant with mtDNA depletion. The authors suggest that mtDNA depletion in HIV-infected but treatment naïve patients may be interpreted as consequence of generalised mitochondrial damage rather than any direct effect on polymerase activity (Miro *et al.* 2004a).

However, it has not been clearly elucidated whether the effect of HIV on mitochondrial function is mostly caused directly by the virus itself or is a consequence of inflammatory mediators released in response to HIV infection. Furthermore, as HIV cannot infect several cell types which have been reported as affected in HIV-infected patients, its effect may be mediated by cytokines at least in neurons, myocytes and adipocytes (Moyle 2005a). Tumour necrosis factor (TNF)- α has been found to induce heart failure associated with mitochondrial impairment in a mouse model. Cardiac muscle cells showed not only a significant reduction in mtDNA when compared with

control animals but also a reduction in the respiratory chain activity and structural abnormalities in mitochondria (Li *et al.* 2001). Similarly, interferon (Inf)- γ has been shown, at least *in vitro*, to be an inducer of mitochondrial dysfunction in smooth muscle cells. The study published by Geng *et al.* shows that Inf- γ induces autocrine secretion of nitric oxide which blocks mitochondrial respiration (Geng *et al.* 1992).

In summary, one could assume that HIV-infected patients might have an HIV-induced sub-clinical mitochondrial dysfunction, which can be exacerbated by the toxic effect of NRTIs. Furthermore, this “double hit” hypothesis on mitochondrial dysfunction in HIV-infected patients is consistent with the finding of advanced HIV disease as a risk factor for both HIV and NRTI-induced complications such as peripheral neuropathy.

In addition, HIV has been shown as an inducer, either directly or indirectly, of intracellular oxidative stress. As described before, NRTIs can also induce oxidative stress and therefore exacerbate the effects induced by HIV infection. It has been described that chronic AZT exposure in murine models can lead to cardiomyopathy in a NADH oxidase-dependent mechanism. That process has been shown to be ameliorated by vitamin C supplementation (Papparella 2007). The effect of antioxidants on NRTI-induced mitochondrial dysfunction has been also studied *in-vitro* showing that supplementation with acetyl-L-carnitine may either protect from the intracellular increase of H_2O_2 (d4T-exposed cells) or reduce the content of H_2O_2 in ddI-treated cells (Ferraresi 2006). Furthermore, N-acetylcysteine, the recognised treatment of choice for paracetamol intoxication, has been able to reverse the oxidative stress induced in a cellular model by HIV antigens (gp 120 and Tat). Therefore, it has been suggested that N-acetylcysteine (Aitio 2005) or perhaps other antioxidants could be used in conjunction with ART to reduce the deleterious effect of both HIV and NRTIs on mitochondrial function. That hypothesis should be tested in a proper way.

CHAPTER 3

Peripheral neuropathy associated with NRTIs

The peripheral nervous system is frequently affected in patients with HIV infection. Different conditions have been described affecting peripheral nerves in the context of HIV disease, some of them are the consequence of opportunistic infections but others have been attributed to the effect of the HIV itself (Table 3.1). Special consideration should be given to the impairment of peripheral nerve function that has been attributed to exposure to NRTIs. This toxic variety of peripheral neuropathy seen in HIV-infected patients, as has been previously mentioned (See chapter 2), has been suggested to be consequence of mitochondrial dysfunction and because of that a study of it has been included in this thesis.

Table 3.1 Peripheral nervous system (PNS) involvement in HIV infection

Modified from Keswani et al. AIDS 2002. 16 (16): 2106

3.1 Cumulative toxicity of NRTIs

Several authors have proposed that NRTI-associated ADR attributable to mitochondrial dysfunction are due to a cumulative toxic effect of these drugs, implying an increasing incidence with longer exposure. A large prospective study assessing subcutaneous fat wasting as a marker of the lipoatrophy syndrome described in HIV-infected patients, has shown that both duration of dual NRTIs therapy before starting HAART and cumulative time on d4T were independently associated with subcutaneous fat loss (Mallal *et al.* 2000). This study showed, in a time-to-event analysis, that the risk of subcutaneous fat wasting was primarily dependent on cumulative exposure to NRTIs. In addition, a few studies on hyperlactataemia in HIV-infected patients treated with NRTIs have suggested that cumulative exposure to NRTIs, particularly to d4T and ddI, may lead to accumulation of mtDNA mutations (Manfredi *et al.* 2003; Coughlan *et al.* 2001).

Early clinical trials for dideoxynucleosides reported data suggesting cumulative toxicity of these drugs. PN was proposed to be a cumulative dose dependent toxicity in phase I clinical trials of ddI and ddC but not d4T. However, the number of patients in these studies was small. All had relatively advanced HIV disease and therefore they might have been at a higher risk of developing PN than most patients currently offered ART (Lambert *et al.* 1990; Petersen *et al.* 1995; Yarchoan *et al.* 1988). In addition, a recently published prospective study on risk factors for PN in HIV-infected people has shown anti-retroviral therapy, particularly dideoxynucleoside exposure, is a predictive factor for PN after a year follow-up. Based on their findings, the authors conclude that it is possible that there may be a threshold of HIV disease after which patients become more susceptible to the toxic effect of drugs and therefore, express clinical evidence of PN. The authors also suggest that such a threshold might be consequence of either duration of the exposure or cumulative dose of ART (Lopez *et al.* 2004).

3.2 Peripheral neuropathy in HIV-infected patients

As mentioned earlier, individuals infected with HIV may present with several types of peripheral nerve impairment. Both opportunistic infections such as CMV and HIV-related processes are mainly seen in patients with advanced HIV disease. By contrast, inflammatory demyelinating polyneuropathies and NRTI-induced neuropathy might be seen at any stage in the course of the HIV disease (Table 3.1).

Although the spectrum of neuropathies described in HIV-infected patients is quite large, the vast majority of patients affected by them present with either HIV-induced distal sensory polyneuropathy or toxic peripheral neuropathy induced by NRTIs (Luciano *et al.* 2003). Interestingly, both conditions are sensory neuropathies and are very similar to each other from a clinical point of view. Given the difficulty of making a differential diagnosis based on clinical information, the temporal association between the initiation of NRTIs therapy and the onset or worsening of symptoms has been claimed to be the most useful distinguishing feature (Keswani *et al.* 2002).

3.2.1 HIV-induced peripheral neuropathy

Peripheral neuropathy (PN) has been shown to be the most frequent neurological complication affecting HIV-infected patients, particularly after the introduction of HAART combinations when the incidence of most opportunistic neurological infections has decreased significantly (Keswani *et al.* 2002; Manji & Miller 2004).

Distal sensory polyneuropathy is usually described as a progressive, symmetrical condition affecting initially the limbs in a centripetal fashion. Patients usually complain of pain or dysesthesia of gradual onset, which might be described as aching, numbness or a burning sensation. This condition is usually more severe on the soles of feet and is particularly distressing at night. On physical examination no evidence of muscular power or tone impairment is

found in the vast majority of cases, the reduction or absence of ankle reflexes being the most evident clinical sign (Keswani *et al.* 2002; Verma 2001).

In the pre-HAART era, distal sensory polyneuropathy was seen in about 35 % of HIV-infected patients, but some autopsy series have shown evidence of peripheral nerve involvement in almost 100 % of cases (Luciano *et al.* 2003; Manji & Miller 2004). In fact, during the period between 1992 and 1995 the incidence of sensory PN increased from 10 to 25 cases per 100 person-years. However, the incidence of PN has decreased gradually since 1996, reaching values around 5 cases/100 person-years (Lichtenstein *et al.* 2005; Lichtenstein *et al.* 2005). Interestingly, the beginning of the rise in the incidence of PN was coincident with the time when NRTIs other than AZT, particularly dideoxynucleosides, were licensed to treat HIV-infected patients (Keswani *et al.* 2002). Nevertheless, the beginning of the subsequent drop in the incidence of PN was evident when HAART combinations were introduced for the treatment of HIV-infected patients (Lichtenstein *et al.* 2005). These data suggest that sensory PN is closely related to both HIV replication and disease status and the toxic effect of dideoxynucleosides.

The exact mechanism by which HIV induces distal sensory polyneuropathy still remains elusive. Nevertheless, a significant association between the probability of developing distal sensory polyneuropathy and progression of HIV disease has been demonstrated (Simpson *et al.* 2002; Childs *et al.* 1999). More immunosuppressed patients are more likely to develop distal sensory polyneuropathy and therefore several factors such as direct HIV effect or indirect damage to peripheral nerves induced by cytokine cascade and even vitamin B12 deficiency, have been proposed as possible causes of distal sensory polyneuropathy (Verma 2001). Pathological studies have shown almost universal peripheral nerve damage in cases with HIV-related death that underwent autopsy.

There is a gradual onset of symptoms in most cases of distal sensory polyneuropathy. These data suggest that the pathology develops gradually and that sub-clinical peripheral nerve impairment might be present in many HIV-infected patients for an undetermined period of time before any symptoms appear (Cherry *et al.* 2003). This is supported by studies showing sub-clinical peripheral nerve dysfunction assessed by both thermal threshold determination and quantitative sensory testing in patients with advanced HIV disease (Tagliati *et al.* 1999; Gulevich *et al.* 1992).

3.2.2 Evidence for NRTI-associated peripheral neuropathy

Phase I clinical trials of ddI, ddC, and d4T demonstrated a clear relationship between dose and the incidence of PN (Yarchoan *et al.* 1988; Browne *et al.* 1993; Lambert *et al.* 1990). In fact, PN was the most important dose-limiting adverse event identified in phase I clinical trials of dideoxynucleosides. In addition, Moore *et al.* have found a synergistic neurotoxic effect when ddI and d4T are given in combination. Moore *et al.* included just over 1,000 patients who were on treatment with either ddI, d4T or a combination of these two NRTIs with or without hydroxyurea. The authors found that those patients exposed to a d4T/ddI combination were 3.5 times more likely to develop PN when compared with those receiving ddI monotherapy. In addition, the combination of d4T/ddI plus hydroxyurea was found to be associated with a hazard ratio of 7.8 for PN compared with ddI monotherapy (95 % CI = 3.92 – 15.5) (Moore *et al.* 2000).

As has been noticed for most NRTI-induced long-term adverse effects, not all dideoxynucleosides have the same capacity for inducing PN. In fact, ddC has been shown as the strongest inducer of PN in clinical studies, followed by d4T and according to some authors, ddI. The incidence of PN in patients receiving ddC at a dosage of 0.75 mg three times a day over a period longer than nine months has been reported to be around 25 %. On the other hand, 17 and 23

% of patients taking d4T at 20 mg and 40 mg twice a day respectively developed PN at week 24 in a large clinical trial (Moyle & Sadler 1998).

Mitochondria have been shown as the target for NRTI-induced toxicities including PN. Dalakas *et al.* have found a much higher proportion of abnormal mitochondria in axons and Schwann cells of sural nerve biopsies from HIV-infected patients treated with ddC when compared with patients with HIV-induced PN or non HIV-infected patients with other causes of PN. In addition, this also showed a significant reduction in the content of mtDNA in the nerve samples of ddC exposed patients compared with controls (Dalakas *et al.* 2001; Cui *et al.* 1997).

In vitro experiments have shown that human neurones (PC-12) exposed to either ddC or ddl concentrations that did not affect cell growth develop mtDNA depletion (Cui *et al.* 1997). Interestingly, the same experiment failed to demonstrate any reduction in the content of mtDNA in neurones exposed to d4T, even at concentrations leading to inhibition of neurite regeneration.

In summary, both clinical and epidemiological data support the association of NRTIs, mainly dideoxynucleosides, and the development of PN. In addition, some in vitro and in vivo studies have demonstrated mitochondrial impairment in patients exposed to dideoxynucleosides.

Assessing ADR in clinical trials is an approach which can generate important information regarding associated clinical and epidemiological factors. However, clinical trials of HIV therapy have been mostly focussed on efficacy rather than safety. Therefore, ADR are usually poorly reported (see section 3.3.3). Nevertheless, clinical trials are considered as the gold standard to assess almost any question in clinical medicine, including those related to safety issues (Bisson *et al.* 2003).

3.3 Delta trial: description and justification

3.3.1 Delta trial

Delta was a large, randomised, double blind, international clinical trial aiming to compare monotherapy against dual combination therapy in HIV-infected individuals (1996). Patients enrolled in Delta were randomised to receive AZT alone or in combination with either ddI or ddC. Patients included in Delta were ART-naïve (Delta 1) or patients previously exposed to AZT (Delta 2). All patients were treated with AZT at 600 mg/day (200 mg three times a day). Those patients randomised to receive ddI were given 400 mg/day (200 mg twice a day), whereas those to be treated with ddC received 2.25 mg/day (0.75 mg three times a day) in addition to their AZT therapy.

Delta was designed with primary endpoints of mortality and AIDS defining illnesses. Nevertheless, safety was also considered and serious adverse events were also defined as secondary endpoints. In that context, peripheral neuropathy was included in the list of expected adverse events and the frequency of PN grade 2 or worse (Table 3.2) was included in the original report published in 1996. Therefore one could expect that PN would be reported consistently through the study period.

A total of 3,207 HIV-infected individuals older than 15 years were recruited for Delta. Overall, 85 % of the participants were male and the mean of age at entry was 36.5 years (SD = 9.3). About 50 % of the study population had 200 or less CD4 cells/ μ l at entry. The median follow-up was 30 months whereas the total follow-up in the study was 7,638 person-years.

3.3.2 Study on peripheral neuropathy in Delta

We performed a sub-analysis of the Delta trial (1996) to further understand the nature of NRTI-associated PN. In particular, the objective of our study was to assess whether the Delta data on PN supported or refuted the hypothesis that NRTI-associated PN, as a marker of NRTI-induced mitochondrial dysfunction, is a cumulative toxic process. In addition, the sub-analysis was also addressed to compare the effects of ddl and ddC.

According to the Delta protocol, assessment of each participant was carried out 4-weekly until week 24 after trial entry and 8-weekly subsequently. At each visit, the case record form elicited whether the patient had developed PN and, if so, the grade of severity on a 4-point scale (Table 3.2). The diagnosis and subsequent classification of PN was primarily based on symptoms suggestive of that condition.

Previous PN was an exclusion criterion for Delta, so none of the included participants in either Delta 1 or 2 had had episodes of PN before being exposed to the study drugs. Regarding other ADR, patients included in Delta 2 should have been exposed to AZT for at least three months before their enrolment in Delta and they should not have developed any major ADR during that previous exposure to AZT to become eligible.

3.3.3 Justification of the sub-analysis

During the first two decades of the HIV epidemic, the main priority in clinical research was to identify therapeutic approaches to treat HIV disease as effectively as possible. Clinical trials therefore have been designed focussing mainly on efficacy rather than safety. In addition, because of the relatively short duration of follow-up in more recent trials, they are not suitable for assessing ADR over the longer term. In fact, only 50 % of the clinical trials on

ART published before 1997 and no more than 15 % of those trials published between 1998 and 2001 presented results after week 48 of follow-up (Ioannidis & Lau 2001). In this context, observational studies may provide the next best level of evidence for longer-term adverse events (Bisson *et al.* 2003).

Nevertheless, HIV-infected individuals are frequently exposed to multiple treatments mainly because combination therapy is the standard of care for HIV disease control and multiple co-morbidities exist. Clinical trials in which randomisation allows blinded administration of pre-determined drug combinations to two or more comparable groups are a valuable source of information on ADR (Bisson *et al.* 2003).

Finally, it has been proposed that the “intention to treat, missing equals failure” (ITT) approach, which is the standard for analysing clinical trials data, might overestimate the tolerability of the drug being tested. ITT analysis usually does not consider changes in the backbone of a given HAART combination neither does it take into account the possibility of patients receiving additional treatment for ART-associated ADR (Carr 2002). Therefore, for clinical trials to address safety questions they must record all relevant information regarding ADR, even if such events do not result in stopping or switching the study medication. It has also been proposed that time-to-event analysis of safety data, through Kaplan Meier plots for instance, might help in adjusting for changes over time in the number of individuals at risk of developing any ADR. In addition, statistical methods such as multivariate Cox regression models might be valuable in adjusting the analysis of risk for a given ADR by potential confounding variables (Bisson *et al.* 2003).

In summary therefore the Delta trial might be considered as an ideal resource to study aspects of NRTI-associated toxicities for a number of reasons. It was a large randomised multi-centre clinical trial with a very long follow-up. In addition, Delta was a double-blind trial so the diagnosis of any ADR was not influenced by the knowledge of which drugs the patients were taking. Finally,

a time-to-event analysis of such a long lasting trial might offer important information on ADR, particularly PN as it was included in the list of the expected ADR in Delta. Therefore, there was the opportunity for participant clinicians to report directly and in a standardised way the occurrence of PN.

3.4 Materials and methods

The primary outcome of this sub-analysis was time from trial entry to first diagnosis of PN, regardless of grade of severity. In this analysis, time was measured from the date of randomisation. All grades of severity were included to allow for the possibility that an initial low grade PN could progress to a higher grade PN and modification of treatment at the initial diagnosis might avoid progression to more severe toxicity (see section 3.5). The median time between randomisation and trial drug prescription was 14 days.

In Delta, there was a high rate of switching from the allocated regimen and data were therefore censored at first change in ART (with a 4-week lag period to allow for any residual drug effect), or at the end of follow-up. Kaplan-Meier methods were used to estimate the cumulative incidence of PN in each of the 3 treatment arms. Nested parametric (exponential and Weibull) survival models were then fitted and likelihood ratio tests used to assess whether the incidence of PN over time was constant. In addition, the effect of the allocated drug regimen on the risk of developing PN using a multivariable Cox model was also assessed, adjusting for updated (most recent) CD4 lymphocyte count, sex, and age at randomisation.

If the association of NRTIs with PN was independent of the effect of HIV on peripheral nerves, one could hypothesise that associations between drug regimen and the risk of PN could be more marked at higher CD4 counts due to a lesser influence of HIV-associated PN. This was investigated by refitting

the multivariable Cox model and including an interaction term between CD4 lymphocyte count (categorised) and allocated drug regimen.

3.5 Results

Of the 3,207 participants recruited in Delta, 12 individuals were excluded from the present analysis (9 never started their allocated treatment regimen, and 3 had no anti-retroviral treatment information recorded). The remaining 3,195 participants had the following baseline characteristics: 1,079 (34 %) were previously treated with AZT for a median duration of 17 months (2 – 72), 85% were male, mean age 36.5 years (SD = 9.3), mean CD4 lymphocyte count 205 cells/mm³ (SD = 114), and 13 % had a previous diagnosis of an AIDS defining illnesses.

After initial randomisation, 1,051 patients were allocated to receive AZT monotherapy whereas 1,076 individuals were randomised to receive dual therapy with AZT and ddI, and 1,068 participants to have dual therapy with AZT and ddC.

Table 3.2 Grade of severity of PN and indication for changing anti-retroviral therapy

Grade	Definition	Resulted in change of therapy			Total (%)
		No	Yes	Not known	
1	Mild symptoms*, no treatment required	55	37	18	110 (62)
2	Moderate symptoms, analgesia required	5	36	5	46 (26)
3	Severe symptoms, narcotic analgesia required	0	7	0	7 (4)
4	Intolerable discomfort, unable to walk despite narcotic analgesia	0	0	0	0 (0)
Not known		9	4	1	14 (8)
Total (%)		69 (39)	84 (47)	24 (14)	177 (100)

* Symptoms: Paresthesiae, pain or numbness

Incidence estimates are based on a total of 4,593 person-years follow-up, a median of 15 months (inter-quartile range –IQR= 6-28 months) per individual. Overall, 177 initial diagnoses of PN were recorded (Table 3.2), which represent an incidence of 38.5 cases per 1,000 person-years (Table 3.3). These diagnoses were most commonly grade 1 (110, 62%) or grade 2 (46, 26%), of which 37 (34%) and 36 (78%) respectively resulted in a modification of ART. All seven cases of grade 3 PN led to ART modification and interestingly, there was no cases of PN grade 4 recorded in Delta. In addition, 47 out of 177 (27%) patients with PN had one or more subsequent diagnoses of PN after their initial diagnosis.

Table 3.3 Incidence of Peripheral Neuropathy by Study Arms

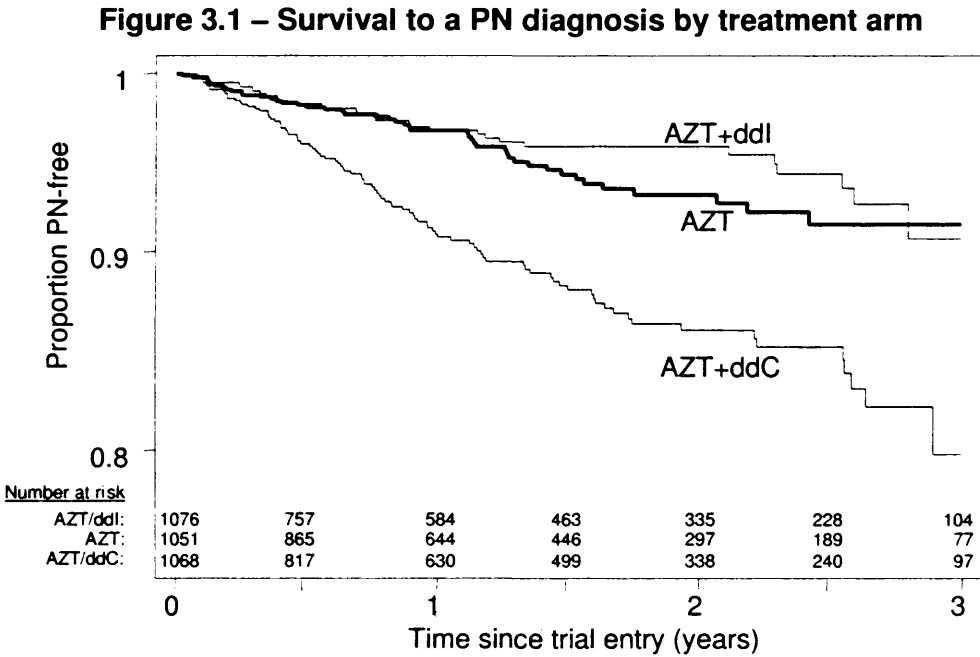
Study Arm	N	Events	P-years	Incidence (per 1000 p-years)	95 % CI
AZT	1,051	46	1,516	30.3	22.7 - 40.5
AZT/ddI	1,076	33	1,497	22.0	15.7 - 31.0
AZT/ddC	1,068	98	1,579	62.1	50.9 - 75.7
TOTAL	3,195	177	4,593	38.5	33.3 - 44.7

Stratifying results by drug regimen received (Table 3.3), PN incidence was substantially higher in patients taking AZT/ddC compared with those on AZT monotherapy (62.1 versus 30.3 cases respectively per 1,000 person-years). By contrast, PN incidence in the AZT/ddI group was slightly lower than that observed among patients on AZT alone (22.0 versus 30.3 cases respectively per 1,000 person-years). However, because confidence intervals overlap the apparent difference between patients treated with AZT monotherapy and AZT/ddI combination may not be real.

In a time-to-event analysis, the incidence of PN appeared to be approximately constant over time for all three study arms (Fig. 3.1). This visual impression was confirmed by significance testing using nested parametric survival models (P = 0.43, 0.27 and 0.19 in the AZT, AZT/ddI and AZT/ddC arms respectively).

Broadly similar results were found in analyses limited to PN diagnoses of grade 2 or higher (results not shown).

Multivariable Cox models were fitted to assess the extent to which comparative differences between drug regimens were mediated by their immunological effects and to explore other risk factors (Table 3.4). After adjusting for current CD4 count, age at randomisation, and sex, the hazard of developing PN was 2.30 times higher in those patients taking AZT/ddC compared with those on AZT monotherapy (95% CI = 1.62 – 3.28, $p < 0.0001$).



There was no significant difference in the risk of developing PN in patients taking AZT/ddl when compared with patients on AZT alone (RR = 0.80, 95% CI = 0.51 – 1.26, $p = 0.34$) (Table 3.4). It is important to notice that 23 individuals were excluded from this part of the analysis as no data on CD4 count were available.

After adjusting for other factors, a low CD4 count and older age were both strongly associated with an increased risk of PN (Table 3.4). Individuals with a

CD4 count of <150 cells/mm³ had a relative risk of developing PN of 2.27 compared with those with CD4 ≥ 350 cells/mm³ (95% CI 1.55-3.34). Patients entering the trial aged 35 years or over had a greater than 2-fold risk of PN compared with younger individuals, but there was no evidence of an effect of gender.

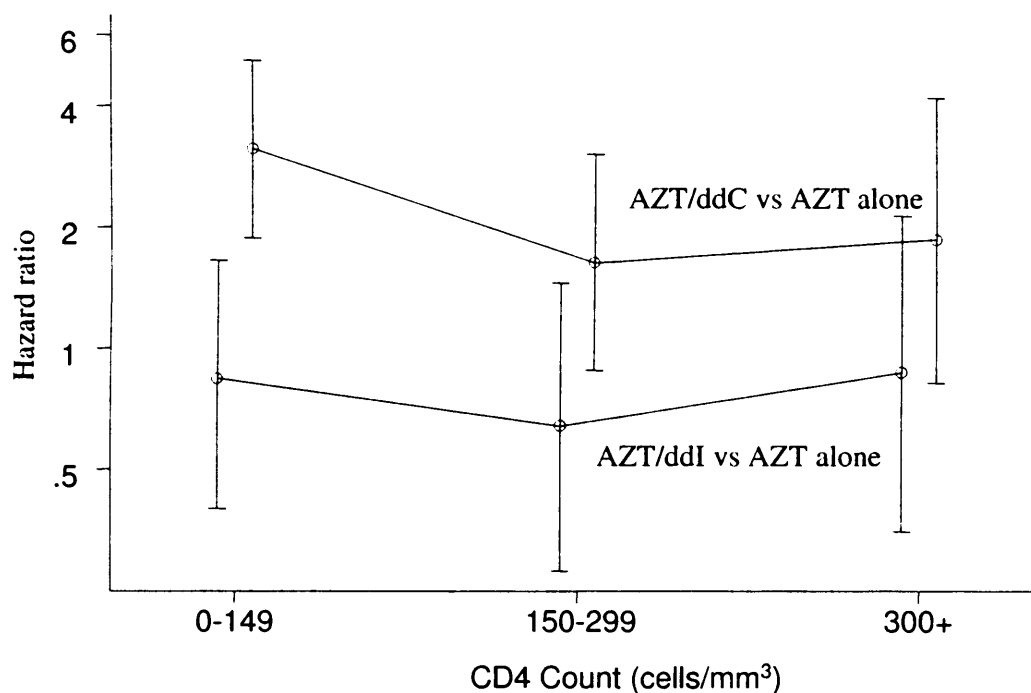
**Table 3.4 Relative risk for Peripheral Neuropathy
(Cox regression model)**

Variable	RH	95 % CI
Allocated regimen		
AZT	1.00	
AZT / ddI	0.80	0.51 - 3.28
AZT / ddC	2.30	1.62 - 3.28
Gender		
Male	1.00	
Female	1.15	0.72 - 1.83
Age at randomisation (years)		
< 30	1.00	
30 - 34	1.13	0.65 - 1.94
35 - 44	2.35	1.46 - 3.80
45 +	2.47	1.49 - 4.11
Current CD4 group		
0 - 149	2.27	1.55 - 3.34
150 - 349	0.99	0.66 - 1.50
350 +	1.00	

An interaction term between CD4 group and treatment arm was added to the model to examine whether the risk of developing PN in patients exposed to either of the study drug combinations was affected by the current (most recent) count of CD4 lymphocytes. The intention behind this was to assess whether CD4 count was an effect modifier for the association between study drug exposure and the risk of developing PN. However, this indicated that the effect of NRTIs therapy on the risk of PN was similar across CD4 strata (Figure 3.2, P-interaction = 0.44), meaning no significant differences were

found in the association between study drugs exposure and the development of PN when controlled by the effect of CD4 count. Therefore, the increased risk of PN in patients taking AZT/ddC compared with those taking AZT alone was consistent in this analysis, regardless of their CD4 count. Similarly, the decreased risk of PN associated with AZT/ddI exposure was also consistent.

Figure 3.2 – Effect of treatment arm on PN risk by CD4 level



3.6 Discussion

In the present study participants who were both naïve (Delta-1) and those with prior AZT exposure (Delta-2) were included to increase the power of the study. This was considered justifiable since AZT monotherapy has not been shown to be associated with an increased risk of PN in several placebo-controlled trials (1994; Hamilton *et al.* 1992; Volberding *et al.* 1990; Richman *et al.* 1987) and no patients in Delta had PN prior to entry.

3.6.1 Evidence for cumulative toxicity

In this study, the incidence of PN was approximately constant over time for all three treatment groups. This observation does not support the concept of a cumulative toxic effect of NRTIs, where one could expect an increase in the incidence of the effect once the cumulative dose reaches a specific threshold. Kelleher *et al.* also failed to find any apparent association between the cumulative ddl dose and incidence of PN in early trials in which the doses used were higher than these currently in use in clinical practice (750 mg/d) and also higher than the doses used in Delta (Kelleher *et al.* 1999). Similarly, in a sub-analysis of the ACTG 175 study Simpson *et al.* present a Kaplan-Meier plot where the incidence of PN also appeared to be constant over time for all study groups (i.e. AZT, ddl, AZT/ddl and AZT/ddC) (Simpson *et al.* 1998). Of interest, the analysis of PN incidence over time included in Simpson's study, which enrolled a smaller number of patients than Delta, included only cases of PN grade 2 or higher. Unfortunately, the precise estimation of follow-up was not reported in the paper, but it was mentioned that patients were followed for about three years. Nevertheless, authors of the last study did not comment on the pattern of incidence of PN over time, but the shape of the presented Kaplan-Meier plot is in agreement with our findings. On the contrary, a time to event analysis presented by Moore *et al.* comparing incidence of PN between patients exposed to ddl monotherapy, d4T monotherapy and a combination of these two NRTIs with or without hydroxyurea (HU), shows a different shape. Although incidence of PN in patients treated with either ddl or d4T monotherapy appear to be constant over time, it is not the case for patients exposed to ddl/d4T or ddl/d4T/Hydroxyurea combinations (Moore *et al.* 2000). Although the authors did not make any comments on it, from the Kaplan-Meier plot one could assume that the probability of developing PN rises abruptly after 10 or 20 months of exposure to ddl/d4T/HU or ddl/d4T respectively.

3.6.2 Incidence of PN by study arms

Our analysis found that the incidence of PN among patients exposed to the AZT/ddI combination was comparable to or slightly lower than the incidence among patients on AZT alone. In Simpson's sub-analysis of the ACTG 175 study, the incidence of PN among those exposed to AZT/ddI and AZT monotherapy was also shown not to be significantly different: 9 % of patients on AZT developed PN compared to 8 % among those on AZT/ddI. Nevertheless, even though this study included similar drug combinations to those used in Delta, the incidence of PN was slightly lower for all of the study arms (i.e. only 11 % of those patients on AZT/ddC developed PN), probably because the study population was different to the population included in Delta. Simpson's study included mainly asymptomatic patients with CD4 counts between 200 and 500 cells/mm³ (Simpson *et al.* 1998).

In our study the incidence of PN of any grade was significantly higher in those patients exposed to AZT/ddC combination therapy compared with those individuals treated with AZT monotherapy, also in agreement with data presented by Simpson *et al.* (Simpson *et al.* 1998). Nevertheless, Kelleher *et al.*, combining data of four randomised phase II/III clinical trials, did not find any difference in the incidence of PN among patients taking ddI, AZT, AZT/ddI and AZT/ddC. However this observation might be confounded by the high dose of ddI used in the trials included in Kelleher's review (600 – 750 mg/day) (Simpson *et al.* 1998; Keswani *et al.* 2002). Interestingly, a pilot study recently published showed that after 48 weeks none of the 24 treatment naïve HIV-infected patients exposed to a combination therapy including AZT/ddC twice a day developed PN (Antunes *et al.* 2004). It is important to highlight that this small group of patients probably had a low risk of developing PN any way because all of them had CD4 lymphocyte count > 200 cells/mm³ and pre-existing PN was an exclusion criterion for that study. In addition, patients enrolled in the Antunes study were also receiving a protease inhibitor and therefore they achieved a better immune response than patients included in Delta.

In Delta, there was some evidence of better clinical response to AZT/ddI than to AZT/ddC. Patients on AZT/ddI had better survival, particularly among those with AIDS at entry in Delta 2 and a longer delay in developing AIDS defining clinical conditions in those without AIDS at entry in Delta 1 compared with similar patients taking AZT/ddC. Survival was also better among those treated with AZT/ddI compared with patients on AZT/ddC in Delta 1 (1996). ART may improve peripheral nerve sensory function in HIV-infected patients (Martin *et al.* 2000). Quantitative assessments of thermal perception thresholds were performed in a group of 49 HIV-infected individuals exposed to ART. The majority of those patients who responded to ART showed improved nerve function during the study period ($P < 0.05$). Those patients, whose thermal perception returned to normal, had higher CD4 counts at entry. Furthermore, individuals with better immune function, induced by ART might be at lower risk of developing PN. One could speculate that in Delta, patients taking AZT/ddI had a lower incidence of PN not only because ddC is a stronger inducer of mitochondrial dysfunction than ddI (Martin *et al.* 1994), but also because patients taking AZT/ddI combination had a better immune response than those taking AZT/ddC.

In a cohort study including over 7,000 HIV-infected patients followed since 1992, the incidence of PN was shown to be inversely correlated with the proportion of patients receiving HAART over time. After 1996, when HAART combinations were introduced, the incidence of PN has decreased steadily. Furthermore, although HAART combinations including drugs such as ddI and low dose (≤ 30 mg twice a day) d4T were associated with PN during the first year of treatment, after a year of exposure any treatment with these drugs became negatively associated with PN (Lichtenstein *et al.* 2005). The last observation suggests that after a lag period immune reconstitution and control of HIV replication induced by ART may reduce significantly the risk of developing PN, even when potential neurotoxic drugs are included in the HAART combination in use.

PN was associated with both dose and duration of ddC and ddI treatment during the phase I clinical trials but not in the expanded access programs with ddI (Lambert *et al.* 1990; Schindzielorz *et al.* 1994; Yarchoan *et al.* 1988). In fact, patients enrolled in expanded access programs with ddI having a history of PN in the past were more likely to develop PN while exposed to ddI when compared with those patients without previous episodes of PN ($P < 0.001$) (Schindzielorz *et al.* 1994). In addition, patients included in both early clinical trials and expanded access programs for NRTIs were usually severely immunosuppressed and this might have implications in patients' susceptibility for developing toxic effects to ART.

3.6.3 Other risk factors for PN

Severity of HIV disease, age and pre-existing neuropathies from any cause have been proposed as risk factors for NRTI-induced PN (Schifitto *et al.* 2002; Chaudhry *et al.* 2003). In almost all studies presented to date, patients with low CD4 lymphocyte count have been found to be at higher risk for the development PN compared with those with less advanced HIV disease (Childs *et al.* 1999; Schifitto *et al.* 2002). In this study we found that the risk for PN appears to increase markedly at CD4 counts below 150 cells/mm³. It has been proposed that NRTIs might be mild neurotoxins capable of unmasking sub-clinical HIV-induced peripheral nerve dysfunction. In a comprehensive review on PN Cherry *et al.* argued that NRTIs cannot induce PN in the absence of an HIV infection background or pre-existing peripheral nerve impairment of any cause (Cherry *et al.* 2003). According to the authors, the absence of reports of PN among individuals exposed to post-exposure prophylaxis (PEP) with dideoxynucleosides might support their point. Nevertheless, the duration of standard PEP regimens (four weeks) might be too short for any toxic effect on peripheral nerves to be expressed. However, in contexts other than HIV infection, toxic PN associated with some chemotherapeutic drugs such as taxol, vincristine and cisplatin has been proposed to be more aggressive in patients with pre-existing PN of any cause (Chaudhry *et al.* 2003).

It has been proposed that HIV-1 envelope glyco-protein 120 (gp120) may induce neuropathic pain and pain hypersensitivity (Oh *et al.* 2001). In addition, inflammatory mediators, such as chemokines (e.g. TNF α produced by activated macrophages) can also induce peripheral nerve dysfunction. This process has been proposed to be Schwann cell mediated and this might explain the axonal degeneration pattern, which has been observed in cases of HIV-induced PN (Keswani *et al.* 2003). HIV-induced distal sensory polyneuropathy, even if sub-clinical, might be a necessary prerequisite for toxic PN associated with dideoxynucleosides.

In a recent large cohort study, HIV-infected patients older than 50 years were more likely to report PN than those individuals younger than 50 years (Zingmond *et al.* 2003). In this study it was found that individuals older than 35 years were 2.35 times more likely to develop PN than individuals younger than 30 years, so the age effect may be broader than has been previously proposed. Age is also a risk factor for other causes of PN such as diabetes mellitus or alcohol abuse (Tapp *et al.* 2003; Ammendola *et al.* 2001). Finally, pre-existing PN has been associated with an increased risk of developing NRTI-associated PN. Even inherited conditions such as hereditary motor and sensory neuropathy or Leber's optic neuropathy can be exacerbated by NRTIs exposure in HIV-infected patients (Miller *et al.* 2002; Shaikh *et al.* 2001; Luzhansky *et al.* 2001). In Delta pre-existing PN was an exclusion criterion, so in this context if previously HIV induced peripheral nerve dysfunction played a part in the development of NRTI-induced PN, this dysfunction had to be sub-clinical.

There was no association between gender and the risk for PN in our study, consistent with previous reports (Lopez *et al.* 2004). However, women appear to be at higher risk of developing other NRTI-associated adverse effects that are believed to be also due to mitochondrial toxicity such as lactic acidosis (Arenas-Pinto *et al.* 2003). Nevertheless, not only in Delta but also in the study published by Lopez *et al.* the proportion of female patients included was low

(about 15 % for both). NRTI-induced mitochondrial dysfunction has been proposed to be organ and drug specific, so it is likely that even when the underlying mechanism is the same, other factors might be crucial in the development of any specific mitochondrial toxicity.

3.6.4 Limitations of this study

Studies on adverse events are usually based on severity scales designed for each complication, which have in common general important limitations. Grade three or four complications (the highest grades in most scales currently in use) do not necessarily predict clinical outcomes and might not have equivalent implications for ART continuation. For instance, nausea grade three normally results in some form of ART modification whereas grade three hypercholesterolemia usually does not imply any acute treatment intervention despite its potential implications for cardiovascular disease (Carr 2002).

Problems related to severity scales are more important when they are exclusively based on clinical information such as the PN gradation scale used in Delta. Furthermore, the assessment of PN-associated symptoms was made by local clinicians without any specialised neurological confirmation of the diagnosis made or involvement in its classification. A consistent degree of discordance between neurologists and HIV doctors regarding PN diagnosis or classification has been observed (Moore *et al.* 2000; Simpson *et al.* 1998). Similarly, physiologic studies on peripheral nerve conduction have demonstrated both the presence of peripheral nerve function impairment in the absence of any clinical symptoms suggestive of PN and normal function of peripheral nerves in patients complaining of neuropathic pain (Tagliati *et al.* 1999).

In summary, one must acknowledge the consequent limitations of the present study. Diagnosis and classification of PN in Delta was made based on a scale of severity including only information regarding symptoms. Furthermore,

clinical signs such as ankle reflex or assessment of sensation were not recorded and no studies on peripheral nerve function were performed. In that context, the reported incidence of PN might not be completely accurate. Severity scales for PN which combine symptoms and signs with or without physiological peripheral nerve function tests might offer a more accurate classification of PN.

In addition, the study records show some variability regarding the classification of the PN diagnosed in a given patient. Some patients with diagnosis of PN were classified as having a high-grade complication at certain point in time and in following visits the classification of the impairment was reduced without switching ART. One possible explanation for this is the possibility of interventions other than analgesia being used to control symptoms, which would not have been recorded. However, we cannot rule out the possibility of changes in the severity classification as a consequence of a different clinician evaluating the patient in subsequent visits or changes in the patient's perception of the severity of symptoms.

Nevertheless, despite the limitations described in the above paragraphs, results of this study are in agreement with previous studies which used more comprehensive case definitions and severity scales (Kelleher *et al.* 1999).

Neither monotherapy nor dual therapy with NRTIs is currently recommended to treat HIV-infected patients any more. However, as has been mentioned before, Delta might be considered as an important tool to assess dideoxynucleoside-associated ADR as it was a large clinical trial with a very long follow-up. In addition, NRTIs, some times including dideoxynucleosides, are currently included in most HAART combinations in use. However, PN has been shown to be significantly associated with the severity of HIV disease. Therefore patients included in Delta might have been more likely to develop PN than patients treated to day as Delta patients had more advanced HIV disease when starting ART and because they could not achieve the level of viral suppression patients on HAART can reach.

3.6.5 Implications for the study on mitochondrial toxicity

PN is a common complication affecting HIV-infected patients and it may be associated with serious disability. Although PN might be induced by NRTIs, in a time to event analysis we did not find any evidence to support the hypothesis of cumulative toxicity. It has also been suggested that NRTI-associated ADR are tissue and drug specific and therefore, pharmacological variables such as drug penetration into specific cells and tissues might affect the likelihood of development of any specific complication after exposure to a given combination therapy including NRTIs.

As described earlier (see Chap. 2), HIV might affect mitochondrial function in treatment naïve patients. Furthermore, ART might result in an improvement in mitochondrial function by controlling HIV replication even when dideoxynucleosides are used in combination with other anti-retrovirals to treat these patients (Miura *et al.* 2003; Casula *et al.* 2004). These observations have been made mainly in PBMC, but taken together with the evidence of peripheral nerve function improvement seen in patients with PN at the beginning of their ART, may suggest a similar positive effect of ART on mitochondrial function in other tissues. Nevertheless, in contrast to other proposed mitochondrial toxicities, clinically evident PN is a common condition in HIV-infected patients. Therefore, if the authors that suggest NRTIs might be mild neurotoxins are right, one could speculate that PN is probably not the best model to assess NRTI-induced mitochondrial toxicity, particularly because of its strong dependency on HIV disease severity.

CHAPTER 4

Systematic review on lactic acidosis in HIV-infected patients

In August 2001 a systematic review of published cases of lactic acidosis in HIV-infected patients was submitted in partial fulfilment as a requirement of the University of London for the award degree of Master of Science in Infection and Health in the Tropics. The project report submitted for the MSc degree also included a proposal for a case-control study on the subject and was conceived as the initial approach to develop a proper research plan for a PhD.

In this chapter I present a summary of what was previously submitted for my MSc followed by a subsequent analysis of the data collected and further preparatory work which was performed at the beginning of the PhD period prior to the final publication. For the analysis of potential risk factors associated with lactic acidosis, such as gender, NRTI exposure allowing for the examination of drug combinations and viral hepatitis co-infection, the original reports were revisited and additional information was collected after submission of the MSc dissertation. The database of the study was redesigned to allow further analysis and the study proforma was amended to be used in the case-control study described in chapters 5 to 7.

4.1 Lactic acidosis and hyperlactataemia

Lactic acidosis (LA) is one of the acid-base disorders included in the category of metabolic acidosis. It may be caused by either excessive production of lactate, diminished clearance capacity of blood lactate or a combination of these two mechanisms. LA has been classified into two different categories:

Type A is the most frequent form of LA and is caused by inadequate oxygen delivery, as in shock or cardiac arrest. The consequence of this condition is an

increase in lactic acid production, but also a reduction in the liver clearance capacity because of reduced hepatic perfusion. Type A LA has been frequently seen among HIV-infected patients, especially associated with septic complications of opportunistic infections.

Type B LA is associated with processes in which tissue hypoxia can not be demonstrated and has been reported in patients with different types of cancer such as lymphoma and leukaemia but also in patients with severe liver failure.

This second type of LA has also been associated with drug-toxicity. Ethanol can produce an increase in the blood lactate level, but is only capable of inducing severe type B LA in the presence of previous hepatic dysfunction. However, other toxic products like salicylates have been identified as a cause of LA (Tantisiriwat *et al.* 2001).

4.1.1 LA and mitochondrial dysfunction

As has been mentioned before (see Chapter 2), most mitochondrial diseases induce LA. This has been seen not only in cases of primary but also in those patients with secondary mitochondrial impairment. Different mechanisms have been identified in the development of LA ranging from genetic alterations leading to aberrant respiratory chain peptides to increased oxidative stress inducing damage to both respiratory chain complexes and mtDNA. Chapter 2 includes a comprehensive review of mechanisms leading to LA in mitochondrial impairment.

4.1.2 Lactic acidosis and hyperlactataemia in HIV-infected patients

Asymptomatic elevated blood lactate concentration has been proposed as a relatively frequent finding in HIV-infected individuals regardless of their exposure to NRTIs or any other ART medication (Vrouenraets *et al.* 2000). In fact, a study on biochemical abnormalities including 1,152 HIV-infected

patients showed that 9 % of them had raised lactate levels (Datta *et al.* 2001). Nevertheless, it has been also demonstrated that the accuracy of the determination of blood lactate is strongly affected by the conditions under which blood samples are obtained and processed, suggesting that at least some mild to moderate cases of hyperlactataemia may be spurious (Andersen *et al.* 2003).

Nonetheless, almost all studies and case-series presented to date suggest that NRTIs, especially d4T, may be significantly associated with the development of severe LA (Tantisiriwat *et al.* 2001; John *et al.* 2001; Gerard *et al.* 2000). In a large study on symptomatic hyperlactataemia it was possible to identify an incidence rate of 0.8% per year of symptomatic cases among patients taking ART (Gerard *et al.* 2000). Interestingly, the incidence of LA increases to 1.2 % per year when only patients exposed to regimens including d4T were considered. However, long-term exposure to NRTIs has been proposed as an important factor for developing both hyperlactataemia (HL) and severe LA (ter Hofstede *et al.* 2000) and d4T has been showed as a marker of long exposure to NRTIs. In fact, Carr *et al.* have reported that most of their cases of LA were on d4T at the time of the event but had received AZT previously. Based on this, they suggested that additive toxicity might increase the risk of developing LA. In contrast John *et al.* established no difference in incidence of LA episodes between AZT-treated and AZT- naïve individuals among patients taking d4T. In John's study, the increased risk attributable to d4T was not confounded by the longer history of NRTIs use (Carr *et al.* 2000; John *et al.* 2001).

The association between HL and LA remain less clear. It has been suggested that symptomatic hyperlactataemia could be an early clinical evidence of progression to LA (Gerard *et al.* 2000). However, LA is reported as a rare complication whereas hyperlactataemia appears to be not an infrequent condition. John *et al.* reported only five cases of severe HL in 516 patients-years of routine lactate measurement (John *et al.* 2001). Nevertheless, of these five patients with blood lactate levels higher than 5 mmol/l only two were

receiving ART at the time of the event and both of them developed LA and eventually died. The calculated incidence rate of NRTI-induced LA in the study presented by John *et al.* was 3.9 cases per 1000 person-years. Nevertheless, a previous study had reported an incidence rate of 1.3 cases per 1000 person-years but this just included exposed individuals (Fortgang *et al.* 1995). Discrepancies on the incidence of LA among HIV-infected individuals exposed to ART are more pronounced if other studies are also considered. For instance, Lonergan *et al.* reported 20.9 cases of symptomatic HL per 1000 person-years on ART. The last study also found that seven out of 10 patients they identified with raised blood lactate levels actually had some evidence of metabolic acidosis (i.e. low blood bicarbonate or high anion-gap) (Lonergan *et al.* 2000).

The incidence of HL or LA may vary depending on the frequency of use of specific NRTIs more likely to induce such complications. However, one additional possible explanation for such discrepancies might be differences in the case definition used by the authors of each report. Unfortunately, there is not a universally accepted classification scale for disorders of lactate metabolism in HIV-infected individuals. Therefore, variations in the cut-off values used to make a diagnosis of HL or the inclusion of clinical data such as symptoms to define a case may make any comparison between studies impossible. In addition, the contribution of other conditions to the development of HL or LA has not been properly assessed. In fact, a study on HIV-infected patients admitted to hospital has reported a prevalence rate of HL about 3 %. Interestingly, the vast majority of these hyperlactataemic patients fulfilled the criteria for LA but most of them were septic at the time and only 15 % of these 27 cases of LA had exposure to NRTIs as the most likely cause of the episode (Tantisiriwat *et al.* 2001). Additionally, in a more recent study all four patients who developed LA had concomitant infectious diseases (i.e. respiratory or urinary tract infections) at the time of the event (Moyle *et al.* 2002). In the last study, the incidence rate of severe HL (Lact > 5mmol/l) and LA was 7.3 and 3.2 per 1000 person-years on ART.

Hepatic steatosis³ has been frequently described in patients diagnosed with LA. In fact, since 1998 there have been frequent reports of cases of “Lactic Acidosis - Hepatic Steatosis Syndrome”. Hepatic steatosis associated with LA in HIV-infected patients has been described as predominantly macrovesicular, but mixed pattern and even microvesicular steatosis as a predominant feature have been reported not infrequently (Lonergan *et al.* 2000; Shaer & Rastegar 2000). Interestingly, liver involvement is frequently seen in patients with severe LA, but not in patients with compensated hyperlactataemia.

Until the first half of 1999 the Food and Drug Administration (FDA), in the US, had received notification of about 60 cases of LA. All these cases were on dual NRTIs therapy, 69 % had hepatic steatosis and 50 % were obese (Revuelta 2000). Other reports have mentioned obesity as a frequent clinical observation in patients with LA (Lonergan *et al.* 2000; Stenzel & Carpenter 2000) but the biggest reviews published recently by Mégarbone and Tantistiriwat did not include obesity in the list of clinical diagnoses more frequently seen in patients with LA (Tantistiriwat *et al.* 2001; Megarbane *et al.* 1999).

Non-alcoholic fatty liver disease (NAFLD) is the most common cause of abnormal liver function test (LFT) results among adults (Day 2002; Angulo 2002) affecting about 20 % of the general population in some settings (Falck-Ytter *et al.* 2001; McCullough 2002). Of interest, cases of NAFLD occur more frequently in female patients (65 – 83 %) with a high prevalence of obesity (60 – 95 %) (Falck-Ytter *et al.* 2001). According to several case-series patients with LA are more likely to be women, be overweight and have previous or concurrent liver diseases such as viral hepatitis B or C.

Despite the lack of conclusive evidence, it is likely that because lactate metabolism is highly dependent on liver function, any severe impairment in liver function may facilitate the development of hyperlactataemia.

³ Hepatic steatosis: Slight to moderate enlargement of the liver due to a diffuse accumulation of neutral fat (triglycerides) in hepatocytes. (Isselbacher and Podolsky. Infiltrative and metabolic diseases affecting the liver. On Harrison's principles of Internal Medicine. 14th Edition. McGraw Hill, New York 1998. pp. 1718.)

Finally, a high case fatality rate (CFR) has been attributed to LA in the context of HIV infection, and in some published series the CFR is 100 %. However, most of these series include a small number of cases and delayed LA diagnosis. Stenzel and Carpenter in their review of complications of ART, suggest that CFR attributable to LA is around 60% (Stenzel & Carpenter 2000).

4.2 Systematic review on lactic acidosis in HIV-infected patients

LA has been identified as a life threatening complication associated with NRTIs therapy. As has been mentioned before, the CFR associated with LA has been shown as extraordinary high reaching 100 % in some small case-series. Nevertheless, conclusive information regarding this complication is lacking.

4.2.1 Justification

Despite being considered as a serious complication, there is limited information regarding the risk factors for LA in HIV-infected patients treated with anti-retrovirals. Most of the available information regarding this issue has been presented in case reports or case-series. In fact, from 1991 to 30th June 2001 there were 217 published cases of LA in medical and scientific indexed journals but 87 of them were presented as part of case series with aggregated data without sufficient individual data on potential risk factors. Additionally, another 40 cases were reported as part of cohort studies examining outcomes other than LA without individual description of cases.

The biggest case-series reported during that period of time included no more than 12 cases whereas the largest reviews of published cases before the one described in this thesis included no more than 60 cases. Interestingly, neither of these two reviews were designed to explore potential risk factors for LA but

aimed to describe clinical presentation of the included patients and to determine factors associated with mortality (Falco *et al.* 2002; Megarbane *et al.* 1999).

Proper investigation of risk factors associated with any ADR requires an appropriately designed study. Nevertheless, the appropriate design of such a study must be based on assumptions grounded in the best available level of information. Therefore, putting together all published clinical information might be the best starting point to assess risk factors for LA in HIV-infected patients.

4.2.2 Materials and methods

An exhaustive search strategy was adopted to identify all published cases of LA in HIV-infected patients exposed to ART. To do so, the following electronic databases were searched:

1. MEDLINE for the years 1982 – 2000 using the search terms lactic acid*, metabolic acid* and HIV infection.
2. AIDSLINE for the years 1982 – 2001 using the search terms lactic acidosis, lactic acidemia, metabolic acidosis, metabolic acidemia and hepatic steatosis.
3. Web of Science v 4.31.
4. The Cochrane Database of Systematic Reviews, Database of Abstracts of Reviews of Effectiveness and the Cochrane Clinical Trials register in the Cochrane Library, Issue 2, 2001. Oxford: Update software.
5. Cab abstracts database.

The abstracts of relevant conferences were reviewed as indexed references at AIDSLINE or by checking available abstract books of scientific or medical events. Additionally, all references cited in included papers were checked and included if pertinent. Selection of reports was not limited by publication source

or language. In fact, three reports written in Spanish, two in French and one in Japanese were included in the review.

Cases were included if they were reported with enough individual description to be entered in the study database. The required individual data included some demographic variables such as age, gender, specific ART received at the time of the event and laboratory values indicating the acid-base metabolism impairment. Cases included in small case-series presented with aggregated data were not included. Patients younger than 16 years old were not included either.

LA was defined using standard criteria: pH < 7.35, serum bicarbonate < 20 mmol/l, or anion-gap > 12 mmol/l in the presence of blood lactate levels > 1.2 mmol/l. In absence of specific numeric data, patients were included as cases if they were defined as “LA cases” by the author of the original report. Patients with diagnosis of clinical conditions potentially leading to LA such as sepsis or alcohol intoxication were excluded, as these were exclusion criteria for most of the original reports.

Duplicate cases were detected by matching variables such as name of the author, hospital where the patient were admitted. Specific variables related to each patient were also matched looking for duplicate cases; these variables include age, sex and date of the LA episode when available.

Relevant information was extracted from each original report using a standardised proforma, which was specifically designed for this review. Data collected were transcribed into a database designed in Access® and subsequently analysed using Stata® version 6. Continuous variables were summarised using the mean and standard deviation if normally distributed or median and range (or inter-quartile range) if not. For categorical data, variables were presented as proportions.

4.2.3 Summary of the data presented as MSc dissertation

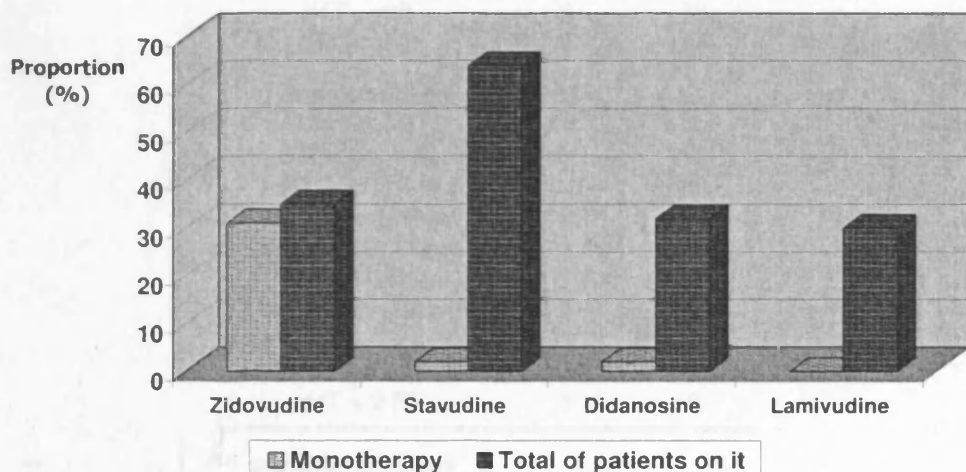
A total of 90 cases with a mean age of 40.11 years (SD=10.38) were included in the review. Of note, 53 % of the cases were female and 6.4 % of them were pregnant at the time of the event. All pregnant women developed the episode of LA during their last trimester of pregnancy.

Information regarding nutritional status was reported in 26 cases of whom 17 (65.4 %) were overweight at the time of the event. Nine out of the 17 overweight patients were female, so there was no gender difference in the presence of overweight ($P = 0.97$).

HIV disease status was not described in 27 cases. Nevertheless, 44/63 patients were reported as fulfilling the criteria for an AIDS diagnosis according to the CDC classification. The median nadir CD4 count was 145 cell/ μ l (IQR = 40 to 210 cell/ μ l) among the 29 cases in whom these data were reported. Furthermore, CD4 count at the time of the LA event was reported in 46 cases and its median was 231.5 cell/ μ l (IQR = 129 to 450 cell/ μ l).

Eleven cases had previous hepatic diseases of which 65 % had viral hepatitis. In addition, seven cases had abnormal results in liver function tests (LFT) before the LA event according to original reports. Hyperlipidemia was reported only in one patient before the event.

Fig. 4.1 Patients on NRTI therapy at the time of the LA event (N = 83)



All 83 patients in whom ART was described at the time of the event were taking at least one NRTIs (Table 4.1). Almost 62 % of the patients were receiving d4T at the time of the event. The median duration of d4T exposure was 8 months (1 – 36 months). Twenty-nine patients (35 %) were taking AZT at the time of the event. The median duration of the AZT exposure before the event was 8 months (1 – 24). Of note, 26 out of the 29 patients on AZT were taking it as monotherapy whereas only two of the 51 patients taking d4T were doing so as monotherapy (Fig. 4.1). Similarly, only two out of the 27 patients who were receiving ddl were taking it as monotherapy. Median duration of the treatment with ddl before the event was 8.5 months (3 – 48). Finally, 27 patients were taking 3TC always in combination with at least one more NRTIs. The median exposure to 3TC before the event was also 8 months (1 – 18). Nevertheless, because of the lack of any comparison or control group, this descriptive analysis offers little conclusive evidence on possible association between ART exposure and LA.

**Table 4.1 Anti-retroviral Therapy
at the time of the LA event
(N = 83)**

ART	Cases	%
Monotherapy	30	
ZDV	26	87
ddl	2	7
d4T	2	7
2 Drugs therapy	16	
ZDV + ddl	2	13
ZDV + d4T	1	6
d4T + ddl	6	38
d4T + 3TC	7	44
3 or more drugs	37	
d4T + ddl + 3TC	1	3
d4T + ddl + PI	12	32
d4T + 3TC + PI	12	32
d4T + ddl + Nev	4	11
d4T + 3TC + Nev	3	8
d4T + 3TC + Del	1	3
d4T + ddl + Efav	1	3
d4T + ddl + PI + Del	1	3
d4T + 3TC + PI + Nev	1	3
d4T + 2 PI	1	3

PI: protease inhibitors

Very few cases had their previous history of ART recorded. However 14 of the 17 cases in whom previous exposure to ART was reported had received AZT whereas seven of them were treated with ddC. Furthermore, adverse effects related to ART were reported in 16/90 patients before the LA event: six cases of myelosuppression, four of myopathy, four of peripheral neuropathy and one case of pancreatitis were reported. Lipodystrophy syndrome was reported as a previous complication in two patients.

Symptoms associated with the event of LA were described in 85 cases and only two cases were reported as asymptomatic. Non-specific gastrointestinal symptoms were the most frequently reported and more than 50 % of the cases had either nausea, vomiting or abdominal pain (Table 4.2). Symptoms classically associated with metabolic acidosis such as dyspnoea, tachypnoea and impairment of consciousness were less frequently described and usually were present in more severe cases.

**Table 4.2 Symptoms Reported
at the time of the LA event**

Symptom	Number of Cases (N = 85)	Proportion (%)
Nausea	45	53
Vomiting	44	52
Abdominal pain	38	45
Weight loss	19	22
Weakness	19	22
Dyspnoea	19	22
Anorexia	16	19
Tachypnoea	13	15
Diarrhoea	8	9
Fatigue	7	8
Abdominal distension	5	6
Impairment of consciousness	2	2
Other	8	9

The median level of blood lactate for the 79 cases in whom the value was recorded was 10.5 mmol/l (range 2.4 and 168.5 mmol/l) (Table 4.3). Arterial blood pH was reported in 52 patients ranging between 7.1 and 7.29 whereas

median blood bicarbonate level was 8 mmol/l (IQR = 5 – 17). Anion-gap was reported in 32 patients with a median value of 25.5 mEq/l (10 – 42). Reported abnormalities in LFT and pancreatic enzymes are presented in table 4.4

Table 4.3 Acid-base status of the patients at the time of the event

Test	Normal Range	N	Median	IQR
Lactate (mmol/l)	0.7 - 1.2	79	10.5	7.0 - 17.3
pH	7.35 - 7.45	52	7.2	7.1 - 7.29
Bicarbonate (mmol/l)	24 - 29	51	8	5.0 - 17.0
Anion-gap (mEq/l)	8.0 - 12.0	32	25.5	18.5 - 31.15

Images suggestive of fatty infiltration of the liver were reported in 13 out of the 20 patients with abdominal ultrasound performed at the time of the event. Similarly, images suggestive of hepatic steatosis were described in 31 of the 32 abdominal CT scan performed at the time of the event. Furthermore, histological evidence of hepatic steatosis was reported in 36 out of the 39 patients who underwent either liver biopsy or autopsy. Microvesicular steatosis was reported in 31 % (8/39) of the tissue examinations whereas 41 % (16/39) of the patients had mixed pattern hepatic steatosis. Twelve patients (31 %) had macrovesicular hepatic steatosis whereas the remaining three biopsies showed hepatic fibrosis and inflammation as their most important abnormality.

Table 4.4 Liver function test and pancreatic enzymes at the time of the lactic acidosis event

Test	Times over upper limit of normal *	Range
Alkaline Phosphatase	1.5	1.04 – 6.6
Alanine aminotransferase	2.5	1.04 – 10.7
Aspartate aminotransferase	3.1	1.2 – 10
Lipase	11.6	1.6 – 787.5
Amylase	2	1.2 – 16.9

* median of

A total of 43 patients had a fatal outcome, which represented 47.8 % of the cases included in this study. Among the 46 non-fatal cases, the median follow-

up was 3 months (0.24 – 30) after the event. In 13 of these cases (28.3%) ART was restarted. Among these patients exposed to a re-challenge with ART, 6 were put on regimens including NRTIs whereas the remaining 7 were challenged with regimens with anti-retroviral other than NRTIs.

4.2.4 Subsequent analysis of the data: the beginning of the work in the thesis period

A second review of the collected data was performed to extract the maximum level of information available regarding specific issues considered important in the process of generating hypothesis on risk factors for LA and to prepare the systematic review for publication. Additional analyses were performed to estimate more accurately the effect of some potential risk factors such as gender (see below).

It was considered important to know where the cases included in the review were diagnosed. It was noticed that reports from three countries accounted for 83 % of the included cases; 56 (62 %) from the United States, 14 (16 %) from France and 5 (6 %) from The Netherlands.

Regarding the ART exposure at the time of the event, it was felt important to describe in more detail the proportion of patients on combination therapy. Thirty cases (36%) were on monotherapy regimens when LA was diagnosed, 26 of them were on AZT. In addition, 16 (19%) were taking two ART drugs (a combination of NRTIs) and 37 (43%) were on combinations of three or more drugs, including either a protease inhibitor (PI) or NNRTIs, at the time of the LA event. One additional patient was taking 3 NRTIs.

Furthermore, no patient was on ABC or ddC at the time LA was diagnosed but among the 17 patients with previous ART regimens reported, 7 had received ddC. Thirteen of these 17 cases were on d4T at the time of the LA event, but all of them had received AZT therapy in the past.

Patients on AZT at the time of the LA event had more advanced disease than those exposed to other NRTIs. The median CD4 cell count among patients on AZT was 79 cell/mm³ (range 18 – 406) (data from 10/29 cases) compared to 282.5 cell/mm³ (range 35 - 1321) (data from 36/61 cases) in those not on AZT.

Regarding past medical history described in 17 patients, who were reported as having had previous hepatic diseases, it was possible to determine that 11 of these were viral hepatitis. Two patients had chronic hepatitis B (HBsAg positive), two hepatitis C (HCV RNA positive), four previous acute hepatitis B and one past hepatitis A. There was no specific information on the two other patients with a history of viral hepatitis. In addition, nine patients were reported as receiving concurrent treatment with potentially hepatotoxic drugs such as fluconazole, ketoconazole or isoniazid.

In 11 patients mitochondrial morphology or function were examined. In nine patients electron microscopy was performed on liver or muscle biopsies or both. In four who had only liver biopsies, enlargement, alteration of shape or increase mitochondrial density were reported (Carr *et al.* 2001; Charton-Bain *et al.* 1997; Khouri & Cushing 2000). In three patients who had muscle biopsies two showed similar mitochondrial ultra-structural changes (Gopinath *et al.* 1992; Sundar *et al.* 1997) and one was reported as normal (Shaer & Rastegar 2000). In one patient who had both a liver biopsy and muscle biopsy, the liver biopsy was abnormal but the muscle biopsy was normal. The remaining patient had a normal muscle biopsy (Olano *et al.* 1995).

Respiratory chain dysfunction was examined in only three cases using spectrophotometric determination of enzymatic complex activity. MtDNA depletion and particularly low levels of activity of complexes 2, 3 and 4 was reported in one case using muscle mitochondria (Roy *et al.* 1999). Two other patients with biochemical evidence of mitochondrial dysfunction were tested using hepatic mitochondria and had reduction of complexes 1 and 1, 3, 4 & 5 respectively (Carr *et al.* 2001; Brivet *et al.* 2000). Finally, mtDNA depletion

was reported in one other patient on muscle and liver biopsy but enzymatic complexes were not examined (Chariot *et al.* 1999).

Regarding the outcome of included cases, in 13/46 (28.3%) non fatal cases it was stated that ART was restarted. NRTIs was used in six of these patients, but the exact duration of follow-up was reported only in three of these six. Three of six patients re-challenged with NRTIs had further episodes of LA. Two were females. One male and one female were challenged with AZT as monotherapy; both developed a further episode of LA but no other adverse effect attributable to NRTIs (Coyle & Abel 1993; Freiman *et al.* 1993). The other female patient was re-challenged with a regimen that was not specified. An additional female patient was re-treated with a combination of 3TC and nelfinavir and followed for 26 months, with no further LA or other adverse event (Shaer & Rastegar 2000). One male patient who developed LA on a combination of d4T and ddI did not resume ART. However, 22 months after discontinuation he developed fatal portal hypertension, liver failure and hepatic mitochondrial dysfunction with normal serum lactate (Carr *et al.* 2001).

4.2.5 Study limitations

Although this is the largest review of published cases of LA to date, it includes only 90 cases and therefore, robust conclusions about the possible risk factors associated with LA are not possible. In addition, it was based on original reports either published in international scientific or medical journals or presented in scientific meetings. Consequently, the review is affected by the expected differences in quality among different reports. Furthermore, it is also affected by the limitations of any retrospective analysis, where missing data is a constant problem, particularly in exercises like this where included cases have been diagnosed over a relatively long period of time. The level of description of cases varied. More recent cases were described in more detail as the level awareness of clinicians was increased by early reports.

Additionally, as the standard care of HIV disease has dramatically changed over a relatively short period of time since the introduction of ART, patients diagnosed at the beginning of the therapeutic era might have been exposed to a different risk factor profile than those who started ART more recently. Patients treated with monotherapy during the late 80's or the first part of the 90's were usually more severely affected by HIV disease and were also less likely to achieve significant and long-lasting viral suppression when compared to patients treated with HAART combinations. This might be particularly important when analysing potential risk factors for NRTI-associated mitochondrial toxicities as it has been demonstrated that HIV itself might affect mitochondrial function (see Chap. 2). Furthermore, advanced HIV disease has been described as an independent risk factor for some NRTI-induced ADR, including peripheral neuropathy (see Chap. 3).

The data included in this systematic review on lactic acidosis was extracted from the original published reports by the principal investigator. The data was entered twice to the database to check for consistency. However, there was not any independent verification of the literature reports.

Nonetheless, the results do add some weight to proposed risk factors from the individual case reports, case series and other smaller reviews. Mégarbane *et al.* described 40 cases published during the 90's accompanied by a literature review of possible causes (Megarbane *et al.* 1999). Falcó *et al.* reviewed 60 cases, and performed a multivariate analysis of possible prognostic factors for mortality (Falco *et al.* 2002). Neither of these reviews aimed to identify risk factors for LA and their literature review strategies were not described.

Finally, our systematic review of published cases was performed to generate hypotheses on potential risk factors for LA. It also aimed to offer the strongest possible grounds for designing a case-control study on this subject.

4.3 Discussion

LA has been recognised as a potentially life threatening complication in HIV-infected patients exposed to ART since the beginning of the 90's. It has been proposed that LA is a relatively infrequent condition (see Chap. 2), but it has been associated with a high mortality. Previous reviewers have reported high CFR associated with LA, of around 60 % (Megarbane *et al.* 1999; Stenzel & Carpenter 2000), but in a more recent study it was 33 % (Falco *et al.* 2002). In our review CFR was 48 % overall. In the first 45 cases, published between 1991 and 1999, the CFR was 64 %. In contrast, for the second 45 cases, published between 2000 and June 2001, it was 33%.

The last paragraph highlights two important issues regarding the complication being studied. Fifty percent of the cases included in this review were published over a period of nine years whereas the second half of cases, the most recent ones, were reported just over a period of 18 months. The lower CFR among more recently published cases might be due to a higher level of awareness among clinicians about LA and earlier identification of cases. Similarly, the increasing level of awareness among clinicians might have encouraged practitioners to report the cases they saw. It is also possible that physicians were more likely to report fatal cases than those who recovered from the event.

4.3.1 Clinical presentation of patients with lactic acidosis

This review highlights that a common presentation of LA is with non-specific gastrointestinal symptoms. Abdominal pain, nausea and vomiting are present in around 50 % of the affected patients (Table 4.2). Of note, similar symptoms have been described in cases of mild to moderate symptomatic hyperlactataemia even in the absence of acidosis. Some authors have suggested that symptomatic hyperlactataemia may be the early and very often non-fatal form of the lactic acidosis-hepatic steatosis syndrome (Penzak & Chuck 2000; Giaquinto *et al.* 2001). Nevertheless, non-specific

gastrointestinal symptoms are quite frequently seen in HIV-infected individuals and therefore a high level of suspicion is required if an early diagnosis of LA is to be made.

In contrast with the moderate evidence of impairment of liver function observed in these patients, hepatomegaly and hepatic steatosis have been consistently reported in patients with LA. Among 90 % of the patients included in this review in whom abdominal imaging studies were carried out, suggestive evidence of liver fatty infiltration was found. Additionally, 36/39 histology examinations showed hepatic steatosis as a predominant feature. Data from this review suggest that macrovesicular steatosis, even in a mixed presentation (i.e. macro and microvesicular steatosis patterns), might be the most important histopathologic finding associated with LA in HIV-infected patients. Another important characteristic is how uncommon the presence of fibrosis or necrosis are in the liver biopsies or autopsy specimens.

Microvesicular steatosis has been associated with drug toxicity, acute liver failure of pregnancy, pancreatic disease, Kwashiorkor and Reye's syndrome (Freiman *et al.* 1993; Sundar *et al.* 1997). Furthermore, microvesicular steatosis is the pattern most frequently seen in patients affected by genetically-induced mitochondrial disorders (Chariot *et al.* 1993). In contrast macrovesicular steatosis is more frequently seen in patients with diabetes, alcohol abuse and steroid therapy. Macrovesicular steatosis is generally associated with low rate of progression to liver failure, but in cases associated with NRTIs the progression to hepatic failure is not infrequent (Sundar *et al.* 1997).

4.3.2 Risk factors for lactic acidosis

LA has been associated with some potential risk factors, but these factors have not been consistently supported by all studies. The case is similar for gender. Some authors have proposed that LA could be more frequently seen in women than in men (Miller *et al.* 2000; Olano *et al.* 1995). However, the

analysis of our series with 90 cases suggests that there may not be a gender difference. 52.8 % of the affected patients were female and the male/female ratio was 0.89. This observation must be analysed with caution since the included cases are from Western Europe, Australia and US. In fact, 83 % of the cases were diagnosed in the US, France and the Netherlands, where the vast majority of the HIV-infected people are male, so similarities in the absolute number of affected women and men could represent a relatively higher proportion of affected women.

Using data on the numbers of HIV-infected individuals receiving care in the US (Bozzette *et al.* 1998), we made a very approximate estimate of the denominator of patients receiving ART, from the estimated number of patients with less than 500 CD4 cell/mm³. Of the total of 209,756 patients likely to be exposed to ART, 44,892 (21 %) were female. Assuming no gender bias in publication of cases, if there were no association between gender and LA, among the 56 USA cases, the expected number of females would be 11, but the observed number was 30. These data suggest that the risk of developing LA could be 2.5 times higher in women than in men (Table 4.5).

Although age has also been suggested as a risk factor for LA, cases of LA have been reported affecting patients of all age groups, including neonates exposed to prophylactic ART (Scalfaro *et al.* 1998; Blanche *et al.* 1999). Carr *et al.* suggested that older patients are more susceptible to develop LA complications and ter Hofstede proposed that this predisposition could be related longer exposure to ART (Carr *et al.* 2000; ter Hofstede *et al.* 2000). However, in our study considering only episodes diagnosed in adults, the range of the age in patients affected by LA is very wide (16 – 69 years of age) and no clusters at any age group could be identified. This would suggest that age might not be an important factor associated with LA.

Table 4.5 Relative Risk of developing Lactic Acidosis by sex

	Number of Pts*	Pts at risk** (CD4 < 500)	LA cases observed	LA cases expected***	RR
Female	52,200	44,892	30	12	2.5
Male	179,200	164,864	26	44	
Total	231,400	209,756	56		

* From: Bozzette SA et al. The Care of HIV-infected Adults in the United States
N Engl J Med 1998;339 (26): 1897-1904

** CD4 < 500 cell/ul (assumed as more likely of receive ART)

*** if risk of LA were the same for men and women

Determining associations between ART exposure and the risk of developing episodes of any ADR is complicated. Firstly, combination therapy is not randomly allocated and secondly patients switch treatments and have long ART exposure histories, which are often poorly recorded. It is therefore difficult to estimate the contribution of individual drugs to a specific toxicity particularly if the event is the result of cumulative exposure to a drug class rather than any individual drug exposure. Nevertheless, a background of HIV infection is not required for NRTIs to produce LA, as there are reports of LA in HIV-negative neonates exposed to prophylactic AZT (Scalfaro *et al.* 1998; Blanche *et al.* 1999).

AZT-associated LA events were reported mainly during the monotherapy era (Megarbane *et al.* 1999). In our series, 90 % of those cases on AZT at the time of the LA episode were taking it as monotherapy. The cases of LA associated with AZT therapy had more advanced disease compared with patients exposed to NRTIs other than AZT. Additionally, the CFR among AZT-treated patients was 68 %, compared with 37 % for patients not exposed to AZT. These differences probably reflect the use of AZT monotherapy in patients with more advanced HIV disease in the late 80's and early 90's as well as the low efficacy of monotherapy compared to combination therapies.

The association between d4T use and hyperlactataemia or LA has been highlighted in previous studies (Brivet *et al.* 2000; Lenzo *et al.* 1997). For

instance, Gérard *et al.* reported a significant increase in the incidence rate of symptomatic hyperlactataemia in patients taking d4T in comparison with patients on other NRTIs from 0.8% to 1.2 % per year (Gerard *et al.* 2000). However, 51 of the 53 patients on d4T in our review were taking it as part of combination therapy, which included at least one additional NRTIs. Furthermore, 24 patients were taking d4T in combination with ddI which, in contrast with 3TC (the other drug associated with d4T), has been implicated as a cause of LA in several reports and has been shown to be an inducer of mitochondrial toxicity in in-vitro studies (Lai *et al.* 1991; Bissuel *et al.* 1994). Cumulative toxicity has been proposed as a risk factor for mitochondrial toxicity and therefore total duration of exposure to NRTIs could be important (ter Hofstede *et al.* 2000). The median duration of NRTIs exposure of patients included in our review was 8 months, but in most cases this period refers to the regimen on which LA occurred and not the total time exposed to NRTIs. If cumulative toxicity is relevant some of the association of LA with d4T might be confounded by a long duration of NRTIs exposure. Previous ART regimens were reported in only 13 of the 52 patients on d4T and all of them had a prior history of AZT use. Similarly, Carr *et al.* found that an important proportion of patients on d4T had received previous treatment with AZT (Carr *et al.* 2000). By contrast, John *et al.* reported that the association between d4T and hyperlactataemia was not confounded by previous treatment with AZT (John *et al.* 2001). The contribution of other NRTIs to the development of LA events is difficult to evaluate because the majority of patients taking ddI or 3TC (92 %) were also taking d4T as it was previously mentioned. Lonergan *et al.* have reported improvements in both laboratory abnormalities and clinical symptoms when d4T was replaced by either ABC or AZT in symptomatic patients with hyperlactataemia (Lonergan *et al.* 2004).

An additive toxic effect between NRTIs and other hepatotoxic drugs may affect the likelihood of developing LA. Olano *et al.* have suggested that the combination of AZT with fluconazole may be a better inducer of LA than AZT alone (Olano *et al.* 1995). In our review nine patients were reported as being concomitantly exposed to fluconazole, ketoconazole or isoniazid. The role of

these drugs has not been evaluated yet, but is not possible to exclude their contribution to the development of LA even though only a small proportion of the patients were using these

Special susceptibility has also been proposed. In this context, it has been proposed that sub-clinical mitochondrial disorders can be unmasked by an NRTIs toxic effect. According to this rationale, previous complications attributable to mitochondrial toxicity could be a good predictor of LA (ter Hofstede *et al.* 2000). In this review, previous adverse events related to ART were reported in only 16/90 cases but all them may be attributable to an NRTIs induced mitochondrial toxicity. Nevertheless, from the data collected in our review, no firm conclusions can be reached regarding this topic.

Previous reviewers have reported high CFR associated with LA, of around 60 % (Megarbane *et al.* 1999; Stenzel & Carpenter 2000), but in a more recent study it was 33 % (Falco *et al.* 2002). In our review the CFR was 48 % overall. In the first 45 cases, published between 1991 and 1999, the CFR was 64 %. In contrast, for the second 45 cases, published between 2000 and June 2001, it was 33%. The lower CFR among more recently published cases might be due to a higher level of awareness among clinicians about LA and earlier identification of cases.

4.3.3 Evidence of mitochondrial dysfunction

In this review, only a small number of cases had investigation of mitochondrial morphology or function. At least 43 studies have shown evidence of NRTI-related mitochondrial toxicity in vitro, and in animal models (Kakuda 2000). As has been described before (see Chap. 2), the proposed mechanism for LA is inhibition of mtDNA polymerase γ leading to mtDNA depletion. However, Walker *et al.* showed that AZT and the combination AZT/3TC can produce raised lactate levels and cell death without a reduction in or deletions of mtDNA in an in vitro study (White 2001). In addition, Bartley *et al.* have reported multiple hepatic mtDNA deletions in a patient with LA (Bartley *et al.*

2001) and recently a case of LA with marked reduction in respiratory chain enzyme activity but not mtDNA depletion has been published. Finally, as has also mentioned in Chapter 2, direct NRTI-induced toxicity on the respiratory chain has also been proposed (Miller *et al.* 2003; Masini *et al.* 1999).

In this review both mtDNA depletion and respiratory chain impairment were described in patients presenting with LA. Nevertheless, because of the small number of cases with mitochondrial studies it is difficult to make any conclusion about the mechanisms involved. Nevertheless, it is clear that all patients had severe mitochondrial dysfunction at the time of their lactic acidosis.

Mitochondrial dysfunction would be expected to lead to an increase in endogenous lactate production. Leclercq *et al.*, using an exogenous lactate challenge test in 11 symptomatic and 3 asymptomatic patients with hyperlactataemia, showed a marked increase in endogenous lactate production even in patients with a moderate increase in serum lactate (Leclercq *et al.* 2001). In addition, in the face of this over-production of lactate, factors which affect lactate clearance such as hepatic dysfunction might also be expected to contribute to hyperlactataemia and lactic acidosis (Brinkman 2000). Concomitant use of drugs such as ribavirin with other NRTIs, i.e. ddI, has also been proposed as a risk factor for the development of mitochondrial dysfunction leading to LA in patients with hepatic dysfunction caused by hepatitis C (Lafeuillade *et al.* 2001).

4.4 Implications of the performed review

4.4.1 Generating hypothesis

LA is likely to be an NRTI-related adverse effect, which affects female patients more frequently. Although it is difficult to evaluate the precise role of individual NRTIs drugs from case reports, it is possible that d4T, and probably also AZT, may be more likely to induce LA when compared with other NRTIs. These

factors may be considered as the strongest hints the review shows in terms of risk factors for LA.

Since the clinical presentation of LA is frequently non-specific and because raised levels of lactate occur in asymptomatic patients, a standard case definition is required to assess risk factors and prognostic factors accurately. In this context, combined case definitions including both biochemical laboratory results and clinical data might be confusing as no correlation has been shown between severity and even presence of symptoms and the level of alteration in biochemical variables. A case definition of LA based exclusively on biochemical laboratory data offers at least two main advantages. Firstly it would be an objective definition and therefore not subject to any inter-observer variation and secondly would offer the possibility of making it comparable to other clinical entities i.e. cases of LA caused by causes other than NRTIs in populations other than HIV-infected individuals.

There are insufficient data to support the effect of other proposed risk factors for LA in this population. Nevertheless, it is possible that factors like concurrent liver impairment or genetic factors such as sub-clinical mitochondrial dysfunction play important roles in the likelihood of developing LA. Furthermore, additive drug toxicity in the setting of a specific sub-group of individuals such as co-infected patients with HIV and HCV exposed to both anti-HIV and anti-HCV therapy including ribavirin may be important (Fleischer *et al.* 2004).

No conclusive information is available on the subject of acid-base metabolism impairment in HIV-infected individuals. Studies are urgently needed to clarify issues such as the proposed progression from mild to moderate hyperlactataemia to severe and life threatening LA and to establish risk factors for that complication.

Hyperlactataemia (HL) and LA may be particularly relevant in the context of an increasing access to ART in resource limited settings, where laboratory

support for early diagnosis might be much weaker than in developed countries. In addition, combination therapies based on NRTIs likely to induce LA such as d4T have been increasingly used in developing countries as the first line suggestion of the World Health Organization (WHO) (World Health Organization 2003). In addition, a significant proportion of patients likely to receive ART in developing countries, particularly in Sub-Saharan Africa, are women and children and the information available on safety issues in these groups is very limited.

CHAPTER 5

Development of the case-control study on lactic acidosis and severe hyperlactataemia in HIV-infected patients treated with anti-retroviral therapy

Following the systematic review of published cases of lactic acidosis in HIV-infected patients exposed to ART (see Chapter 4), a number of questions on risk factors for such a complication became evident. Many variables had been proposed as associated with LA, but conclusive evidence of these proposed associations were lacking for most of them.

As has been mentioned before, most of the available information regarding LA in this type of population has been derived from case reports or case-series, which by definition cannot provide definite information on risk factors for any suspected drug-induced complication. Therefore, it was critically important to design and perform studies addressed to identify risk factors for LA in HIV-infected patients.

5.1 Justification for a case-control study

LA has been described as an infrequent complication attributed to ART and therefore, identifying risk factors for that using any other epidemiological study design would be impractical. Case-control study design has been shown to be useful for studying rare or infrequent outcomes, and therefore has been used to investigate other rare but serious adverse drug reactions (ADR) (Strom 2000). Case-control studies on the other hand have been described as not appropriate for rare exposures, unless exposure often leads to the outcome of interest. Nevertheless, this not the case of many drug adverse events and particularly is not the case for LA in this population as the complication has been associated with NRTIs exposure and this drug family is by far the most frequently used in the treatment of HIV infection.

In addition, case-control studies allow the simultaneous assessment of multiple exposures that are potentially associated with the outcome of interest. As has been mentioned before (see chapter 4), several variables and exposures have been proposed as risk factors for this complication.

Furthermore, case-control study design is particularly convenient when the latency period between exposure and outcome is relatively long. This feature is particularly relevant to this study as there is not conclusive evidence of the relevance of long exposure to ART and the development of LA. It has been suggested, but not proven, that longer exposures are associated with higher risk for this and other ART-induced mitochondrial dysfunction among HIV-infected individuals (Brinkman *et al.* 1998). In addition, the case-control design may also be convenient to assess the relevance of total ART exposure rather than current drug exposure for the event to occur.

No temporal relationships between any exposure and the outcome can be derived from case-control studies. Therefore, no causality association can be demonstrated but the strength of an association between a specific exposure and the outcome can be estimated. Furthermore, associations confirmed in any case-control study between exposures or risk factors and the outcome are significant as hypothesis generating data. Furthermore, at least some exposures may be assumed to be certainly present before the outcome. This is particularly the case for studies assessing drug exposures as potential risk factors for acute and objectively defined outcomes.

Nevertheless, case-control studies have significant weaknesses which must be taken into account. Case-control studies are prone to bias and that is one of the most important limitations of this type of epidemiological study. With retrospective studies, recall and reporting bias may critically affect the accuracy of the results. However, when information for the study is collected directly from a source produced at the time of the event rather than from what either included participants or health care workers can recall some time after

the outcome occurred, the likelihood of these types of bias affecting the accuracy of the results is much lower.

Other important potential source of errors in designing case-control studies is the process of selection of participants. It is critically important to develop a clear and unambiguous case definition. Differences in the reported incidence of either LA or HL among different studies may be partially explained by the use of different case definitions. In addition, presence of symptoms may not correlate precisely with the level of blood lactate making it difficult to include both laboratory markers and symptoms, potentially associated with the complication, in a case definition. The use of objective criteria to define a case may prevent getting a definition that is too wide and less specific and this may lead to more conclusive and accurate results even if some cases are not identified. For metabolic complications such as LA and HL it was assumed that using appropriate thresholds for laboratory variables may lead to an appropriate case definition. Furthermore, the lack of electronic databases in some centres may force one to rely on clinicians' memories to identify potential cases. Such a practice may introduce important bias in the study as some specific patients may be more easily remembered; for example, those patients more seriously ill or those diagnosed in the context of specific conditions such as clinical trials.

Selection of controls is also a critical step in the development of a case-control study. Controls are meant to represent the distribution of the exposure of interest in the study population where cases are identified. In this context, controls must have been likely to have had similar exposure to ART than cases or at least controls must be at similar risk of being prescribed the drugs prescribed to cases. As prescription patterns have changed over time, selection of cases and controls for this study must take into account such a variation in exposure likelihood (see section 5.3.4).

In addition, it is important to acknowledge other limitations of retrospective studies. The level of awareness of LA and HL as complications of ART has

also changed over time. Therefore the intensity of monitoring for these complications as part of regular care offered to HIV-infected individuals on ART has changed as well. In this regard, not only the number of investigations performed on patients to diagnose these complications but also the level of description of the events and interventions indicated to treat or prevent it may have also changed over time. As a result, the level of available information about cases recorded in clinical notes may also vary over time. Furthermore, monitoring of blood lactate or any other relevant laboratory variable may not be a routine practice in all participant centres.

It may be obvious that prospective, randomised clinical trials may be the best possible strategy to ensure that all participants are going to be under equal diagnostic surveillance and therefore the most likely study design to produce clean and conclusive data on risk factors associated with a given outcome. Moreover, given the high frequency of co-morbidities seen in HIV-infected patients and the increasing offer of available therapeutic options randomisation and blinded administration of predefined therapeutic combination might be the only way to control for all known and unknown confounders. Clinical trials therefore, may be the most appropriate strategy to identify associations between ART drugs and long-term adverse events (Bisson *et al.* 2003). Unfortunately, such trials may not be possible for many reasons, including logistic complications related to the long follow-up period required, associated costs and lack of interest of the pharmaceutical industry. In addition to that, it may also have ethical implications as randomisation to exposures thought to be more harmful may not be acceptable.

Therefore, observational studies and particularly case-control studies can offer a good opportunity of generating valid and conclusive data on risk factors associated with drug-related adverse events. In fact, case-control studies have been performed in different settings to assess safety questions related to many drugs and some of them have impacted clinical practice quite significantly. A good example of the potential benefit of this study design in assessing drug safety is the study on diethylstilbestrol and vaginal

adenocarcinoma published by Herbst in 1971 (Herbst *et al.* 1971). The study included only eight patients with clear-cell adenocarcinoma of the vagina who were matched to four controls each and it showed a very strong association between maternal exposure to estrogens during the first trimester of pregnancy and vaginal cancer in their daughters.

5.2 Background

As has been highlighted before (see chapter 4), most of the initial published data on LA were based on case reports or case series including few cases, the largest one being a series published by Mégarbane in 1999 which included 40 previously published cases (Megarbane *et al.* 1999). However, in the following years, a few observational and retrospective studies were performed addressing several questions on LA in HIV-infected individuals exposed to ART.

A few cross-sectional studies have identified and therefore described cases of LA. Moyle *et al.* managed to identify four cases of LA among 108 individuals with raised blood lactate levels (Moyle *et al.* 2002) whereas the Swiss Cohort Study has reported only one case of LA among 73 patients with hyperlactataemia (Boubaker *et al.* 2001). A small case-control study addressed to identify risk factors for LA has also been published. The study published by Bonnet *et al.* in 2003 included nine cases of LA (Bonnet *et al.* 2003). Interestingly, all nine cases reported by Bonnet were severely ill and all were admitted to an intensive care unit.

The identification of risk factors for case fatality in HIV-infected patients who develop LA was the aim of a study published by Falcó *et al.* The study included a series of cases (N=12) diagnosed and managed by authors and a group of 60 previously published cases (Falco *et al.* 2002). That study did not address the question of risk factors for LA itself.

5.2.1 Proposed risk factors for lactic acidosis

All cases of LA included in the systematic review we have performed were receiving at least one NRTIs at the time of the event and about 97 % of them were on a thymidine analogue (i.e. zidovudine or stavudine) at the time when the event was diagnosed (see chapter 4). Similarly, the review published by Mégarbane also highlights that the vast majority (83 %) of the included cases were receiving a thymidine analogue at the time of the event (Megarbane *et al.* 1999). Eleven out of 12 cases of LA reported by Falcó were taking d4T either as monotherapy or in combination with one or more NRTIs at the time of the event.

Nevertheless, in the study published by Moyle *et al.* in 2002 current exposure to ddl was over-represented among patients with either severe HL or LA (Moyle *et al.* 2002). Similarly, the Swiss Cohort Study has also highlighted the potential role of ddl exposure as risk factor for HL. Of note, the results presented by the Swiss Cohort Study suggest that the higher risk attributable to ddl may be more pronounced when this drug is used in combination with d4T. The authors found that the risk of developing HL may be significantly higher (HR=2.7, 95 % CI= 1.5 – 4.8) amongst those patients on d4T with or without ddl when compared with those patients on AZT based combinations (Boubaker *et al.* 2001).

Furthermore, a matched case-control study (N= 21 cases) addressed to evaluate risk factors for HL has reported that d4T exposure is a significant independent predictor of sustained HL (adjusted hazard ratio = 4.27, 95% CI = 1.02 – 17.79) (Datta *et al.* 2003). Exposure to ddl was one of the matching factors. In addition, it is important to mention that the case definition they used was based on a blood lactate threshold of 3.5 mmol/l, whereas the cut-off used in most studies for severe HL is 5 mmol/l.

As has been discussed in chapter 4, variables other than NRTIs exposure have also been proposed as risk factors for LA. It has been suggested for

instance that female patients are at higher risk of developing LA when compared to men. Based on the data we collected in the systematic review presented in chapter 4, the risk of developing LA in women exposed to ART may be more than twice higher than for men. In fact, the Food and Drugs Administration (FDA) in the United States produced a warning letter a few years ago highlighting the increased risk seen in women exposed to ddI/d4T combination therapy. Similarly, in a study performed in London (which included nine cases with blood lactate higher than 5 mmol/l, four of whom were acidotic), female gender was overrepresented among the cases group (Moyle *et al.* 2002). Nevertheless, the study published by Moyle *et al.* failed to demonstrate any significant association between female gender and the likelihood of developing HL (defined as blood lactate higher than 2.5 mmol/l) (Relative Hazard= 1.39; 95% CI= 0.74 – 2.61).

Previous or concurrent NRTI-induced adverse events have also been proposed as risk factors for LA. Previous and sustained mitochondrial impairment affecting different tissues or organs has been proposed to increase the likelihood of developing life threatening LA (Carr & Cooper 2000; Manfredi *et al.* 2003). Similarly, advanced HIV disease and low CD4 nadir have been proposed as risk factors for almost all mitochondrial toxicities related to NRTIs, including LA (Bonnet *et al.* 2003; Anderson *et al.* 2003).

Liver diseases, including not only viral infections but also other conditions such as non alcoholic fatty liver disease have been proposed to be associated with an increased risk of developing both symptomatic HL and LA in HIV-infected individuals (John *et al.* 2001; Gerard *et al.* 2000; Dagan *et al.* 2002).

5.2.2 Lactic acidosis and severe hyperlactataemia

Over the past few years a few observational studies and case series have addressed several questions on proposed NRTI-associated mitochondrial toxicities. These studies have used several models from PN to peripheral lipodystrophy to assess the impact of specific drug exposures on these

outcomes. However, as has been mentioned before, several other factors apart from NRTI-induced mitochondrial dysfunction may play significant roles in almost all proposed mitochondrial toxicities. Nevertheless, hyperlactataemia has been recognised as a marker of mitochondrial dysfunction in many clinical situations in the general population. Therefore, HL and or LA may be the ideal outcome to evaluate the effect of any ART drug on mitochondrial function.

LA is a condition well described in several medical fields and its definition is universally accepted. Patients with elevated blood lactate, usually above 2 mmol/l (or 18 mg/dl), low arterial blood pH (< 7.35) and low blood bicarbonate (< 20 mmol/l) are recognized as cases of LA. It is possible however to identify patients with high blood lactate and low blood bicarbonate who do not have low arterial blood pH. In that condition the bicarbonate buffer is efficient enough to keep blood pH in the normal range in the presence of a harmful factor leading to an imbalance in the acid-base metabolism. Patients with high blood lactate and low blood bicarbonate may be in an earlier stage in the process which will eventually lead to LA if the noxious agent is not controlled.

In addition, arterial pH has not been regularly measured in HIV-infected patients with abnormal blood lactate who have been included in most of the studies published to date. Therefore, a high proportion of the available information is on cases of HL. In fact, the management recommendations on metabolic complications associated with ART for HIV-1 infection published in 2002 by the International AIDS Society-USA Panel suggested using the term lactic acidemia instead of LA or HL to describe HIV-infected patients with elevated lactate (Schambelan *et al.* 2002). Furthermore, it has been proposed that even when measurement of arterial pH confirms the diagnosis of LA, it may not be necessary in many instances assuming that severe HL is itself a diagnostic criterion for lactic acidemia (Schambelan *et al.* 2002; Carr 2003). Following the rationale previously described, a diagnosis of lactic acidemia based on the level of blood lactate and regardless of the blood pH may be an indication for a medical intervention anyway. This implies a progression from

HL to LA and therefore assumes that controlling episodes of HL will prevent its evolution to LA.

In addition, it has also been suggested that the diagnosis of NRTI-associated lactic acidemia requires the exclusion of other known causes of raised blood lactate such as dehydration, vigorous exercise, sepsis, hypoxemia, alcohol intoxication, renal failure, hyperthyroidism and other drug-induced HL (Carr 2003).

Although there is not a universally accepted definition of severe NRTI-induced lactic acidemia, some diagnostic criteria have been proposed, most of them include clinical symptoms in the definitions. The IAS-USA Panel suggested that a diagnosis of NRTI-induced lactic acidemia can be made in the presence of either 1) a confirmed blood lactate above 5 mmol/l (45 mg/dl) plus new “related” symptoms or signs or 2) blood lactate above 10 mmol/l (90 mg/dl) regardless of the clinical presentation (Schambelan *et al.* 2002). Similarly, Powderly in a review published in 2002 includes the category of symptomatic lactic acidemia but acknowledged that there is a separate category which he called LA with hepatic steatosis (Powderly 2002). In addition, Powderly includes a separate category for patients with asymptomatic lactic acidemia and/or chronic HL, as a condition not including acidosis. As has been mentioned before (see Chapter 4), mild to moderate HL may be a relatively frequent condition among HIV-infected patients exposed to NRTI-containing combinations. Nevertheless, it has also been proposed that these mild elevations in blood lactate in asymptomatic patients may be either transient and of uncertain clinical significance (Powderly 2002) or may be just spurious results as a consequence of several possible mistakes in the process of taking or processing blood samples.

To get accurate measurements of blood lactate, patients must be instructed to not perform vigorous exercises for 24 hours before the test and must be well hydrated at the time of blood sample collection. In addition, samples must be collected without use of tourniquet or fist clenching in a pre-chilled fluoride-

oxalate tube. The sample must then be transported on ice to the laboratory, where it must be processed within four hours of collection (Carr 2003). If samples are not taken and processed in the right way, the results may be spurious showing an elevation, generally mild, of blood lactate which is not a real reflection of the metabolic status of the patient. Therefore, some authors have suggested that most if not all cases of asymptomatic mild HL (blood lactate between 2.2 and 5 mmol/l) are due to spurious results rather than a true effect of any drug on lactate metabolism (Andersen *et al.* 2003).

Conversely, confirmed blood lactate results above 5 mmol/l (45 mg/dl) are likely to correspond to real cases of sustained and severe lactic acidemia, even if arterial blood pH is not measured. The latest assertion is based on the rationale described in the paragraphs above but has not really been demonstrated in the context of HIV infection and NRTIs exposure.

Finally, as has been mentioned before, symptoms claimed to be associated with LA and HL are non-specific and difficult to classify. In addition, symptoms such as abdominal pain, nausea, fatigue, weakness, etc are frequently seen in HIV-infected patients taking ART. Taking both together, it is likely that the precision of the description of these symptoms in clinical notes would not be very high and therefore, in the context of a retrospective study, it would be difficult to assess accurately not only the presence but also the severity and duration of the symptoms, making it difficult to use clinical information for the case definition. In addition, it has not been demonstrated that there is any correlation between presence or severity of any clinical variable and the severity or prognosis of the event of either HL or LA (Walmsley *et al.* 2004).

For the present study it has been considered pertinent to combine HL and LA as a single outcome based not only on suggestions and assumptions made by experts in the field but also because biological plausibility of the progression from HL to LA. In addition, logistical issues were also taken into consideration. It was considered that if HL and LA are steps in a continuous process, given the low incidence described for LA, combining the two entities would increase

the probability of collecting a significant number of cases in a relatively short period of time. In addition, clinical practice had changed in many centres, by the time of our study, and patients were generally switched away from specific ART drugs when an episode of HL was diagnosed. This would make it even harder to find cases of LA if HL progresses to LA without intervention.

5.3 Study description

5.3.1 Aim of the study

The main motivation for this study was to identify risk factors associated with LA and HL in HIV-infected individuals exposed to ART. Particularly interesting to us was the proposed association between NRTIs exposure and these complications. Therefore, the study was designed to identify and quantify the strength of any association between any specific NRTIs or NRTIs combination and LA or HL. The study would pay special attention to history of ART exposure as well as the drug or drug combination patients were taking at the time of the event.

In addition to drug-related variables, other factors would also be tested as potential risk factors for LA and HL. Such additional factors would include demographic parameters as well as previous medical conditions either related or not to HIV infection as is described in section 5.6.4.

Although the study was designed to identify risk factors for LA and HL, it would be also the largest case-series ever compiled on the subject and because of that, provide a detailed descriptive analysis of epidemiological, clinical and biochemical characteristics of cases compared to controls.

Finally, as LA had been described to be associated with a high mortality rate, an analysis on risk factors for case fatality would also be possible.

5.3.2 Study design

A retrospective case-control study was designed to achieve the aims described above. Because of the proposed relatively low incidence rate of LA among HIV-infected patients exposed to ART (see Chapter 4), an international multi-centre study was considered as the best possible option to include a sufficient number of cases to reach convincing conclusions in a relatively short period of time. The study therefore, was planned to include cases and controls regularly seen in specialised centres from Europe, the Americas and Australia.

The study included confirmed HIV-infected adult individuals receiving or previously exposed to ART. Cases were to have biochemical evidence of either lactic acidosis or severe and sustained hyperlactataemia, whereas controls were to be randomly selected from patients attending the same clinics or admitted to the same hospital wards as the cases.

5.3.3 Case definition

Following the rationale discussed previously (see section 5.2.2) both cases of lactic acidosis and severe and sustained hyperlactataemia were eligible to be included in the study. It is important to mention that both entities are defined solely in terms of biochemical markers, so no clinical data are considered for any of the case definitions.

- I. Cases of lactic acidosis: Out or in-patients meeting the following criteria were considered eligible for the study. The criteria used in this study to define lactic acidosis are in agreement with the definition widely used in medical practice.

- Arterial blood pH < 7.35
- Blood bicarbonate < 20 mmol/l (22 mEq/l)
- Blood lactate > 2.2 mmol/l (19.8 mg/dl)

Patients had to meet all three criteria to be included as cases. Nevertheless, discrepancies between the cut-off previously mentioned and the reference ranges used by centre laboratories did occur. Cases of lactic acidosis in that situation were defined following the same principle (i.e. low arterial pH, low blood bicarbonate and high blood lactate) but taking the cut-off from the reference normal range for each specific laboratory.

II. Cases of severe hyperlactataemia: Irrespective of their acid-base metabolic status, patients with at least two consecutive readings of blood lactate above 5 mmol/l (45 mg/dl) were considered eligible as cases.

III. Controls: HIV-infected patients seen as outpatients or admitted to hospital at the same centres as the included cases. Controls, as well as cases, were older than 16 years and being exposed to ART, even if they were not on treatment at the time of being selected as participants in this study.

Two randomly selected individuals, meeting the above-described criteria, were selected as controls for each included case.

5.3.4 Matching criteria

Because of the lack of conclusive information regarding variables associated with either LA or HL it was assumed that matching, as a strategy to control for confounders, might exclude the possibility of detecting important associations. One might argue that it would be logical to attempt to design a study where controls are randomly selected from the entire study population and not matched at all to cases by any variable. It is likely that a simple study design like this would produce clean results which may be valid as long as the sample size was large enough.

Nevertheless, apart from the logistic complications which may be associated with using the entire study population as a source of controls, there are important methodological and scientific reasons to include at least some matching variables in this study. First, ART drugs have been developed and introduced into clinical practice at different points in time over the last 15 years, and therefore the probability of being exposed to a given drug or drug combination varies with time. Patients who developed the study outcomes during the late 90's were unlikely to be exposed to the same drug combinations as individuals who started ART several years later. Therefore, to make cases and controls comparable with respect to the likelihood of being exposed to specific ART drugs, it was decided to match cases and controls by calendar time.

In addition, patterns of ART prescription may vary not only as function of time but also according to the centre where patients are regularly treated for their HIV infection. Countries and centres have their own guidelines and so specific ART drug combinations may be more frequently prescribed in a given centre. Therefore, the likelihood of exposure to any specific ART drug combination or even the threshold for switching therapy might differ between participant centres. As a result it was also decided to match cases and controls by centre. So controls were matched to their respective cases by time and place.

The criterion used for matching by time was calendar year. Therefore, controls were to be patients receiving regular outpatient care or admitted to hospital during the same calendar year when their respective case was diagnosed with either LA or HL. Despite the dramatic changes we have seen in prescription patterns of ART drugs over the last few years, a calendar year was assumed as an appropriate period because changes are usually gradually introduced into clinical practice and so it was felt that a shorter time frame would not necessarily improve the precision of matching.

The controls were selected from individuals who had been seen at the same clinic at least once or admitted to the same hospital ward during the year when their respective case was diagnosed with either LA or HL.

5.4.4 Ethical issues

After a comprehensive discussion with invited researchers, the study protocol was submitted to all relevant ethics committees for approval. In the UK the protocol was submitted at separate times to two different committees, the Local Research Ethics Committee (LREC) of Camden & Islington Community Health Services NHS Trust for the pilot study and later to the Multi-Centre Research Ethics Committee (MREC) of London for the full study in UK centres. Full approval was granted to the study for both entities.

This was to be a retrospective study designed to collect information already recorded at each participant clinical notes without requiring any direct contact between participants and investigators. However, in the UK individual informed consent from each living participant in the UK was required. Furthermore, according to the ethics committee's conditions, each participant was to be consented by his or her regular physician, preventing the principal investigator from approaching any potential participant directly. Explicit permission from each relevant NHS trust Caldicott Guardian⁴ was required to include any deceased patient in the study.

Separate patient information sheets about the study as well as consent forms were prepared for cases and controls. All these documents were prepared following LREC and MREC guidelines and were approved by the relevant body before being used.

⁴ NHS Caldicott Guardians: Senior staff in the NHS and social services appointed to protect patient information (Definition taken from the DH website, available at www.dh.gov.uk/PolicyAndGuidance/InformationPolicy/PatientConfidentialityAndCaldicottGuardians/ , accessed on 8th June 2006)

Proformas used to collect the relevant information for the study were anonymous, but included a unique identification number. The local investigator at each locality was the custodian of a list linking the study number and patients' identification data.

Similarly, ethical approval was obtained from all relevant ethics committees in all participant countries. Identification and inclusion of patients in the study in each international centre was performed in accordance with the conditions imposed by the relevant ethics committees. Not all centres required consent. Where individual consent was required local investigators were the custodians of the consent forms signed by each participant.

5.5 Pilot study

A pilot study was carried out. The aim of the pilot study was to identify potential pitfalls and gaps in the instruments to be used for the case-control study such as the proforma and database and to amend such instruments and study procedures. The pilot also assessed the time required to consent patients, complete proformas and transcribe the data onto the database.

5.5.1 Centres involved

The pilot study was conducted at the Centre for Sexual Health and HIV Research, University College London and Camden Primary Care Trust (PCT). The study therefore included outpatients regularly seen at the Bloomsbury Clinic, Mortimer Market Centre.

Patients admitted to the Patrick Manson Unit at University College Hospital, (University College London Hospitals NHS Foundation Trust) were also eligible for the study. Finally, patients seen at Archway Sexual Health Clinic, also part of Camden PCT were also included in the pilot study if they met the inclusion criteria.

5.5.2 Identification of cases

Cases diagnosed between 1990 and 2003 were identified using different methods. First, discharge summaries from the Patrick Manson Unit were searched for diagnoses of acidosis, lactic acidosis, and lactic acidemia. In addition, those discharge summaries reporting abnormal blood lactate levels were also checked. Some patients who met the study case definition among those with abnormal lactate reported in their discharge summaries had diagnoses such as mitochondrial toxicity, pancreatitis, peripheral neuropathy, and sepsis. Consequently, we searched the discharge summaries again to assess whether or not patients discharged with those diagnoses met the study criteria to be included as cases.

In addition, the Bloomsbury and Archway Clinics database was searched also for laboratory results. Specifically, blood lactate and bicarbonate values were searched to identify eligible patients for the study. The search on laboratory results was made using the threshold values previously defined (see section 5.3.3). As arterial pH is not recorded in the database, it was not used as a searching variable. Nevertheless, pH values were checked in all patients identified through the other strategies described.

Finally, all clinicians working at both sites were asked about any potential cases they remembered. A few cases were identified using this approach but all of them were also caught by the other mechanisms.

5.5.3 Selection of controls

Controls were selected from a list of eligible patients. The list was obtained by selecting people seen in clinic at any time during the period of interest according to the diagnosis date of each case. According to the study protocol, that period corresponded to the calendar year when each case was diagnosed. However, as people seen in clinic during a year period would be likely to include all the clinic population and most patients are seen in clinic

every three or four months, the list of eligible controls for each case was limited to a three month period including one month before and one month after the month when each case was diagnosed.

Then, each list was filtered by ART exposure. Following the study protocol the list was filtered not by current exposure but exposure to ART at any time. Therefore, the final list included only patients eligible to become controls for the study. The list was later completed adding all those patients who were admitted to Patrick Manson Unit over the same period of time but who were not seen in clinic as outpatients. Careful cross check was performed to avoid duplications and to ensure that every patient in the list was included just once.

Finally, controls were randomly selected from these merged lists. A hand calculator was used for random number generation which was used to select controls for each identified case.

5.5.4 Findings

Fifteen cases were identified and included. Other five potential cases were not included as they did not meet the inclusion criteria in full. Eight out of the 15 included cases met the criteria for LA whereas the other seven had HL. Two of the cases had died but only one as a consequence of the episode of LA. Five cases were identified as cases of LA through discharge summaries, whereas the other three cases of LA were identified by checking blood lactate levels and arterial blood pH in patients with diagnoses such as pancreatitis, peripheral neuropathy and bacterial infections such as sinusitis. All the cases of HL were identified through a database search for blood lactate results. Three out of the seven cases of HL were also picked up from the discharge summaries search, where one of them was also classified as a case of mitochondrial toxicity.

Once the cases were identified, two controls per case were randomly selected as previously described (see section 5.5.3). Both cases and controls were

approached by their regular doctors by post. Each potential participant received an invitation letter signed by his or her regular doctor plus a copy of the patient information sheet and three copies of the consent form printed in a Camden PCT NHS Trust headed paper. The package also included a pre-paid addressed envelope for patients to return two of the signed consent forms if they agreed to take part in the study. The consent form patients signed clearly stated that only information already recorded in their medical notes or records was going to be used for research.

Over a two month period the response rate was higher than 90 % among cases who were alive but it was only 54 % among invited controls. Because of the poor response rate among controls it was considered necessary to implement a second strategy. A copy of their invitation letter as well as copies of the correspondent patient information sheet and the consent form were placed in each patient set of notes for patients to be consented during their following consultation. The process of getting consent from controls using the second approach took five months but led 90 % of the consents not obtained by the first method being obtained. Only two potential controls declined to take part in the study (7 %). They were replaced by two additional patients who were selected in the same way as original controls were. One case was also consented using this second approach. The entire time period required to consent all participants in the pilot study was seven months.

Relevant information both cases and controls was collected in a standardised way using the same proforma. The study proforma was designed on the basis of the data collected during the systematic review (see Chapter 4) and divided into three sections (as described for the database on section 5.6.2). For cases the index date was the date when the diagnosis of either LA or HL was made according to the study case definition, regardless of the date of admission if the case was an inpatient (or the date when the clinician made the diagnosis). For controls the date of the consultation each control had closest in time to the date when the respective case was diagnosed, was chosen as the index date. Therefore, all information collected for controls is referred to the date of one

specific consultation held during the calendar year when the respective case was diagnosed with either LA or HL.

5.6 Case-control study design

Although the aims of the study remained the same, after analysing the experience of the pilot some adjustments were made in the study design to facilitate its applicability. Therefore, the following section describes these amendments and details the analysis strategy for the case-control study.

5.6.1 Objectives

In the light of both the systematic review (see Chapter 4) and the pilot study results it was agreed to focus the case-control study to achieve the following objectives:

- I. To describe the demographic and clinical characteristics of HIV-infected patients with lactic acidosis and severe hyperlactataemia.
- II. To describe the biochemical characteristics of these patients at the time of diagnosis and their evolution.
- III. To identify risk factors associated with lactic acidosis and severe hyperlactataemia.
- IV. To identify factors associated with case fatality in those patients affected by either lactic acidosis or severe hyperlactataemia.
- V. To compare risk factors for lactic acidosis and for hyperlactataemia as separate outcomes.

5.6.2 Sample size calculation

From the systematic review on published cases on lactic acidosis we found that treatment with d4T might be more likely to induce LA than other NRTIs exposures (see Chapter 4). Therefore treatment with d4T was assumed to be the main exposure of interest for this study. Furthermore, using retrospective data from the Mortimer Market Centre we estimated that about 40 % of the clinic population may have been exposed to d4T over the study period (September 1990 to December 2004). We therefore used that figure to calculate the sample size we would need for this study assuming that 40 % of the control population may have been exposed to d4T over the study period.

Because of the lack of previous information on the study outcome it was decided empirically to design a study with a 90% power of achieving significance at the 5% level to detect an odds ratio of two or more for the risk of LA or HL from the use of d4T.

Applying the formula proposed by Kirkwood for sample size calculations (Kirkwood 1988) (Fig. 5.1) it was estimated that the minimum sample size required for the study was 135 cases plus 2 controls per case. Therefore, the total study population aimed to be recruited was at least 405 participants.

Fig. 5.1 Formula used for the sample size calculation

$$N > \frac{\{u \sqrt{[\pi (1 - \pi)]} + v \sqrt{[\pi_o (1 - \pi_o)]}\}^2}{(\pi - \pi_o)^2}$$

5.6.3 Patients and methods

Patients diagnosed between 1990 and 2004 and regularly seen at seven centres in the UK and 12 international centres were included. UK centres were mainly located in London (King's College Hospital, Chelsea & Westminster

Hospital, North Middlesex Hospital, Northwick Park Hospital) but centres located in other regions of the country i.e. Brighton and Manchester were also included. Table 5.1 shows a list of all participant centres, including international ones.

Table 5.1 Participant centres

Centre	Location
King's College Hospital	London, UK
North Middlesex Hospital	London, UK
Northwick Park Hospital	London, UK
Chelsea & Westminster Hospital	London, UK
Mortimer Market Centre, UCL/ Camden PCT	London, UK
Brighton General Hospital	Brighton, UK
North Manchester General Hospital	Manchester, UK
Academic Medical Centre	Amsterdam, The Netherlands
University Hospital	Zurich, Switzerland
Hospital Clinic	Barcelona , Spain
Hospital Carlos III	Madrid, Spain
St Jame's Hospital	Dublin, Ireland
Hvidovre Hospital	Copenhagen , Denmark
Rigshospitalet	Copenhagen , Denmark
Fundación Huesped	Buenos Aires, Argentina
Policlínica Metropolitana	Caracas, Venezuela
Washington University, St Louis	St Louis, USA
Alfred Hospital	Melbourne, Australia
St Vincent's Hospital	Sydney, Australia

Most participant centres had electronic databases which were used to identify cases and to select controls. Procedures vary from centre to centre, but the basic principles were always observed. Laboratory results were the main searching tool, being mostly based on blood lactate and bicarbonate levels given the study thresholds. As was described in the section on the pilot study, pH values were almost never recorded in databases, so patients' notes had to

be checked to assess their eligibility. In those centres where patients had two sets of notes, as outpatients and inpatients, both sets were searched.

Controls were selected following the study protocol specifications. Each centre produced a list of eligible patients, mostly using electronic databases to do so. Where electronic records were not available a list was produced using clinic booking records. As these records often did not include information on treatment exposure, the clinical notes of each randomly selected patient were checked to ensure eligibility.

Different procedures were followed in two specific participant centres. In one international centre (St. Vincent's Hospital, Sydney) all patients booked for clinics during a three weeks period were considered as potential controls and consented during that specific consultation. Most of the patients seen in clinics during that period of time were actually consented. Finally, the number of needed controls according to the number of cases identified in that centre was randomly selected from that group of pre-consented patients. Nevertheless, the index date for matching was consistent with the study design.

The other centre (Fundación Huesped, Buenos Aires) is mainly a clinical research unit and therefore, all patients seen there were involved in at least one clinical trial. Furthermore, during specific periods of time the number of clinical trials enrolling patients was relatively small. As a consequence of that, the population of patients to select controls from was relatively small compared to other participant centres. A special procedure was carried out to generate a list of eligible patients by combining the available lists of participants in each clinical trial. The intention was to not limit the group of available potential controls to those patients enrolled in the same trial to avoid an indirect matching by ART.

In an attempt to increase consistency of the data and to reduce inter-observer variability it was planned that all study forms from centres would be completed on site by the principal investigator. Although this was fulfilled in some centres

(53 % of the included centres), mainly in the UK, it was considered not appropriate in some other centres. Legal regulations, administrative procedures and in some cases language limitations prevented the principal investigator to having access to the actual clinical notes and therefore in such centres local investigators were responsible for completing the study proformas. In those centres where study proformas were completed by local researchers (37 %), the principal investigator carried out an audit-style review of the study proformas in situ to check for consistency and to completeness of the recorded information. All inconsistencies or missing data identified by the principal investigator were immediately reviewed by the local researcher against the actual clinical records.

Although the principal investigator was involved directly in the data collection at most participant centres, there were two centres, one UK and one international, which were not visited by the principal investigator. The UK centre was visited by a clinical research fellow from the Centre for Sexual Health & HIV Research, who was trained in the procedures followed by the principal investigator to either complete or audit study proformas. The additional international centre was included in the study after reviewing the study proformas, which were completed and brought to London by a local investigator. The requested additional information was either obtained from additional documents the local researcher brought with them to London or sent electronically to the principal investigator a few days after the audit meeting.

All collected data were transcribed into a database designed in Access[®] in a systematic way. The accuracy of the transcribed information was assessed by three different mechanisms. Firstly, for numeric variables included in the database all valid entries were specified using validation rules (e.g. for variables coded as 0, 1 and 2, only these figures were allowed as valid answers). Secondly, after transcription, the data was randomly audited. Fifteen percent of the cases were checked against the information collected in the study proformas. During the last process, errors were found in no more

than seven records and almost all of them were minor discrepancies in dates. As a result, the last mechanism to check data accuracy was performed. A separate table including demographics and ART drug history variables was produced to manually check consistency of the original database.

5.6.4 Analysis strategy

The primary analysis was planned before the data collection phase and it was conceived to test specific prior hypotheses which generated during the systematic review of published cases of LA (see Chapter 4). Nevertheless, it was acknowledged that several secondary analyses would become pertinent after completing the primary analysis. In order to pursue these it was decided that the database and the study completed proformas will remain at the Centre for Sexual Health & HIV Research. Any proposed secondary analysis should be approved by the study steering committee, which includes not only the principal investigator and researchers from the co-ordinating centre but also local investigators involved in the study. This thesis is restricted to the primary analysis which is summarised in table 5.2.

Statistical analysis was performed using STATA version 9.1(StataCorp LP, College Station, Texas USA). Categorical variables are presented as proportions. Medians and inter-quartile range (IQR) are used to present continuous variables, as most of them were not normally distributed. Significance was assessed using likelihood ratio test (LR) for categorical variables. The same test was performed to the log10 transformation of continuous variables. Differences in duration of exposures to NRTIs were assessed using Mann-Whitney test. Significance testing included in this study takes into account the matching variables. Conditional logistic regression was used to identify risk factors associated with the study outcome. A multivariate model was built using the stepwise forward approach. The criterion used to be included in the multivariate model was a $P < 0.05$ in the LR test for the univariate analysis. Comparisons between LA and HL were done considering the matching variables. Finally, classical logistic regression was used to

identify risk factors for case fatality. Again, the model was also built using the stepwise forward approach.

For the analysis of factors associated with fatality and the likelihood of developing any further episode of HL/LA categorical variables were presented as proportions and significance tested for by using χ^2 test. Continuous variables were summarised as median and IQR and significance assessed with the T test. Logistic regression models were developed to identify factors associated with fatal outcome and further episodes of HL/LA.

5.6.5 Definitions and assumptions for the case-control study

For this study the following definitions were used.

- Base-line: refers to CD4 count or HIV viral load. Before any ART exposure.
- Current exposure to ART: Patients receiving drugs at the time of the event or at the time of being included in the study. Patients who stopped ART because of the symptoms associated with the event within a two week period before the diagnosis were also included in this category.
- Previous exposure to ART: Any exposure to ART drugs patients may have had before the combination they were taking at the time of being included in the study.
- Current ART duration: Duration of the exposure to ART drugs patients were taking at the time of inclusion in the study.
- Past ART duration: Duration of ART exposures patients may have had before the combination therapy taken at the time of their inclusion in the study.
- Total ART duration: Duration of all exposure periods to any ART drug patients may have had. Generally includes past and current exposures to a given drug.

- Past medical event: Refers to OI or ADR. Event diagnosed before the last regular follow-up consultation each patient had before the event was diagnosed in cases or the index date for controls.
- Concurrent medical event: Refers to OI or ADR. Event diagnosed after the last regular follow-up consultation each patient had before the event was diagnosed in cases or the index date for controls.
- Chronic hepatitis B or C infection: Patients with HBsAg or AntiHCV positive results for at least six months. Patients explicitly described as Hep B or Hep C/HIV co-infected in their clinical notes.

Table 5.2 Variables assessed in the primary analysis

I. Demographics
I.1. Age at the time of inclusion
I.2 Gender
I.3 Ethnic background
I.4 Country of birth
II. HIV disease history
II.1 Time known as HIV positive
II.2 Most likely mode of HIV infection
II.3 Previous AIDS diagnosis
II.4 Nadir CD4 count (previous to ART exposure)
II.5 Peak HIV viral load (previous to ART exposure)
II.6 Previous ART-related adverse drug reactions
II.7 ART exposure complete history
II.7.1 Current exposure to any specific ART drug
II.7.1 Previous exposure to any ART drug
II.7.3 Duration of the exposure to any ART drug
III. Past medical history
III.1 Chronic hepatic diseases
III.2 Viral hepatitis B and C markers
III.3 Diabetes mellitus
III.4 Cancer (non HIV-related)
III.5 Drug exposure
III.5.1 Including recreational drugs and drugs associated with LA
IV. Event of interest
IV.1 Symptoms
IV.2 Imaging and other tests performed
IV.3 Laboratory tests performed
IV.3.1 Acid-base metabolism
IV.3.2 White blood cell count and haemoglobin
IV.3.3 Liver function tests
IV.3.4 Blood glucose, amylase and lipase
IV.3.5 Blood urea and creatinine
IV.3.6 Blood lipids
IV.4 Concurrent opportunistic infections
IV.5 Concurrent ART-associated adverse drug reactions
IV.6 Management
IV.7 Outcome
IV.8 Further ART exposure
IV.9 Further episodes of LA or HL
V. Comparison between LA and HL
V.1 Risk factors for LA
V.2 Risk factors for HL

CHAPTER 6

Results of the case-control study on Severe hyperlactataemia and lactic acidosis

6.1 Introduction

As described in chapter 5, the main objective of this study was to identify risk factors for HL/LA. However, because this is the largest case series on HL/LA compiled to date, a descriptive analysis is presented as introduction to this chapter. In addition, risk factors for HL/LA are analysed considering ART exposure as the main variable to be assessed. Nevertheless, all other non-pharmacological factors associated with the study outcome are analysed as well.

6.2 Identification of risk factors for hyperlactataemia and lactic acidosis

6.2.1 Descriptive analysis of included patients

A total of 110 cases and 220 controls were included in the study. The geographical distribution of the study participants is shown in table 6.1 but briefly, the majority (64%) of included cases were patients regularly seen in the European participant clinics. Forty nine of the cases had LA and the remaining 61 cases had HL.

The proportion of males included as cases was 63.6 % whereas the proportion of male controls was 81.8 %. Cases were significantly older than controls ($P=0.011$). The median age of cases was 42.4 years (IQR=36.01 - 52.5) whereas for controls it was 40 years (IQR= 35.0 – 47.1). Stratifying age at the time of the event by gender, we found that male cases were also significantly older than controls (median age 43.1 and 41.5 years respectively; $P=0.010$).

Table 6.1 Cases included in the study by region

Region	LA*	HL*	TOTAL
United Kindom	16	16	32
Continental Europe	16	23	39
North America	4	8	12
South America	5	4	9
Australia	8	10	18
TOTAL	49 (45%)	61 (55%)	110

* LA= Lactic acidosis; HL= Hyperlactatemia

Table 6.2 shows some additional demographic characteristics of the study participants. Female gender was overrepresented in the case group as 36.4 % of them were female compared to only 18.2 % of controls ($P < 0.001$). Three pregnant women were included in the study, one was a case. The majority of the study population (65.3 %) was white (a category which includes white Europeans, Americans and Australians). Nevertheless, the proportion of non-white cases (including black African, black Caribbean, black American, Hispanics and Asians) (41.8 %) was significantly higher than among controls (30.8 %) ($P=0.014$). It is important to mention that not all study centres document ethnicity in all patients. In fact, a few centres do not record ethnicity at all. Furthermore, there is little ethnic variability among the study population; most study participants were white or black (89.3 %) meaning that just about 10 % of the study population belongs to a different ethnic background (i.e. Hispanic or Asian). In addition, a significant ethnic homogeneity was noted in most of participant centres populations. For instance, in centres such as Sydney, Madrid, Barcelona and Caracas all or almost all study participants belong to a single ethnic group. Therefore, as all the analyses presented in this chapter take into consideration matching variables and because the health centre is one of them, given the homogeneity of some centres population, matching by centre may be an overmatching by ethnicity.

Cases were more likely to have been infected with HIV through exposures other than homosexual contact ($P= 0.009$). Fifty nine percent of cases were

likely to be infected either by heterosexual exposure (40 %), intra venous drug use (14 %) or blood transfusion (5 %) whereas about 63 % of controls were likely to be infected through homosexual exposure. It may be obvious that this difference between cases and controls may be confounded by the higher proportion of female patients in the case group compared to the controls. However, it may also be affected by the differences in the ethnic background of study participants, since 70 % of male cases and controls were white, whereas 60% of female cases were non-white, compared to 50% of female controls (Table 6.3).

Table 6.2 Demographic characteristics of included patients

Variable	Cases		Controls		P
	N	%	N	%	
Demographics					< 0.001
Male*	70	63.6	180	81.8	
Female	40	36.4	40	18.2	
Mode of infection					0.009
Homosexual*	41	41.0	125	62.8	
Heterosexual	40	40.0	47	23.6	
IVDU	14	14.0	23	11.6	
Blood transfusion	5	5.0	4	2.0	
Ethnicity					0.014
White all*	50	58.1	108	69.2	
Black all	22	25.6	36	23.1	
Asian	5	5.8	2	1.3	
Hispanic	9	10.5	10	6.4	

* base-line category

Eleven cases (10 %) had a previous diagnosis of diabetes mellitus (DM) compared to two controls (0.9 %) ($P < 0.001$). Similarly, proportionally more cases (22.7 %) than controls (11.4 %) had a diagnosis of high alcohol consumption ($P = 0.004$). Furthermore, cases were also more likely to have

pre-existent hepatic disorders other than viral hepatitis. Nine cases (8.2 %) had either hepatic steatosis (2/9), alcohol related hepatitis or cirrhosis (2/9), non-alcohol related cirrhosis (1/9) or hepatitis of unknown aetiology (4/9) whereas only 5 controls had any of the previous hepatic conditions mentioned before ($P= 0.018$).

Table 6.3 Ethnic background of study participants

Variable	Cases (N= 86)				Controls (N= 157)			
	Male		Female		Male		Female	
	N	%	N	%	N	%	N	%
White*	36	70.6	14	40.0	92	74.2	16	50.0
Black**	7	13.7	15	42.9	24	19.4	12	37.5
Hispanic	5	9.8	4	11.4	6	4.8	4	12.5
Asian	3	5.6	2	5.7	2	1.6	0	0.0
Other	0	0.0	0	0.0	1	0.8	0	0.0

* Includes white Europeans, Americans and Australians

** Includes black African, American and Caribbean

6.2.1.1 Severity and markers of HIV disease

Information regarding the date of the first positive HIV serology test was available from 107 cases (97 %) and 216 controls (98 %). The median duration of the period between the first positive result and the date of inclusion in the study was not significantly different between cases and controls, 70.1 months in cases (IQR= 20.6 – 132.3) and 82.2 months in controls (IQR= 39.2 – 130.3) ($P=0.515$).

Nevertheless, cases were more likely to have more advanced HIV disease when compared to controls both at baseline (i.e. before any exposure to ART) and at the time of the LA/HL event. Almost 54 % of cases had a history of an AIDS defining condition compared to only 37.2 % of controls ($P= 0.004$) (Table 6.4).

The median nadir CD4 count (previous to any exposure to ART) among the 91/110 cases in whom such a value was available was 120 cell/ μ l (IQR=50 - 200). The median nadir CD4 count for 188/220 controls was 180 cell/ μ l (IQR= 60 – 285) (P= 0.011) (Table 6.5).

Table 6.4 Previous AIDS defining conditions

	Cases (N= 110)		Controls (N= 220)		Overall
	N° Events	%	N° Events	%	%
Candidiasis	15	13.6	24	10.9	11.8
Cryptococcosis	8	7.3	3	1.4	3.3
Cryptosporidiosis	1	0.9	5	2.3	1.82
CMV infection	7	6.4	8	3.6	4.6
HIV encephalopathy	4	3.6	2	0.9	1.82
HSV infection	11	10.0	14	6.4	7.6
Histoplasmosis	1	0.9	2	0.9	0.9
Isosporiosis	1	0.9	0	0.0	0.3
Kaposi's Sarcoma	11	10.0	22	10.0	10.0
Lymphoma	9	8.2	4	1.8	3.9
Mycobacterium non TB	6	5.5	13	5.9	5.8
Tuberculosis	6	5.5	3	1.4	2.7
Pneumocystosis	17	15.5	30	13.6	14.2
PML	1	0.9	0	0.0	0.3
Toxoplasmosis	6	5.5	5	2.3	3.3
Wasting syndrome	4	3.6	6	2.7	3.0
Recurrent pneumonia	2	1.8	4	1.8	1.8
Any AIDS defining condition	59	53.6	82	37.3	42.7

Although there were only a small number of episodes of opportunistic infections (OI) at the time of the event, cases were more likely to have a concurrent AIDS defining illness when compared to controls. Nineteen cases (17.3 %) and 18 controls (5.2 %) had a concurrent OI at the time of inclusion (P= 0.015).

These differences between cases and controls are not affected when HIV-related variables are stratified by gender. Approximately half of male and female cases (52.9 and 55 % respectively) had previous AIDS diagnoses. The

median nadir CD4 count for 60 male cases was 130 cell/ μ l (IQR= 56.5 – 235) and 100 cell/ μ l (IQR= 26 – 156) for the group of 31 female cases with information on CD4 count. The median CD4 count at the time of the HL/LA event was 220 cell/ μ l (IQR= 122 – 370) for male cases and 252 cell/ μ l for female cases (IQR= 150 – 300). Both male and female controls had current CD4 counts about 400 cell/ μ l (400 and 389 cell/ μ ml respectively).

6.2.1.2 NRTIs exposure

Cases were much more likely to have been exposed to either d4T or ddI at any point in the course of their illnesses than controls. In fact, 92.7 % and 77.3 % of cases had ever been exposed to d4T and ddI respectively compared to 68.6 % ($P < 0.001$) and 53.6 % of the controls ($P < 0.001$). Exposure to AZT or 3TC was more frequently seen among controls than cases and such differences were also highly significant ($P = 0.004$ and < 0.001 respectively).

Duration of NRTIs exposure was not significantly different between cases and controls except for d4T. Cases were more likely to have been exposed to d4T for shorter periods than controls. Fig 6.1 shows the median, IQR and range of current d4T duration by study group. The median duration of the current d4T exposure for cases ($N = 73$) was 11.74 months (IQR= 8.62 – 24.47) and 18.42 months for controls ($N = 79$) (IQR= 8.32 – 40.33). Such a difference in the duration of the current exposure to d4T was not statistically significant (Mann-Whitney test $P = 0.061$), but a clear trend was noticed.

The presence of possible outliers was explored to see if the difference in duration of the exposure to d4T was driven by controls exposed to that drug for extremely long periods. Unexpectedly outliers with longer duration of exposure were identified in the cases rather than in the controls. Figure 6.1 shows six observations which lay outside the range expected for the distribution and all of them were cases.

Table 6.5 Surrogate markers of HIV disease severity

Variable	Cases			Controls			P
	N	Median	IQR* (Range)	N	Median	IQR* (Range)	
HIV duration (months)**	107	70.07	20.56-132.6 (4.9-218.5)	216	82.24	39.18-130.33 (1.1-229.5)	0.175
Nadir CD4	91	120	50 - 200 (2 - 660)	188	180	60 - 285 (1 - 714)	0.011
Current CD4***	100	248	136.5 - 365 (3 - 884)	212	400	220 - 551.5 (1 - 1596)	< 0.001

* IQR= interquartile range

** Time known as HIV positive

*** at the time of the event

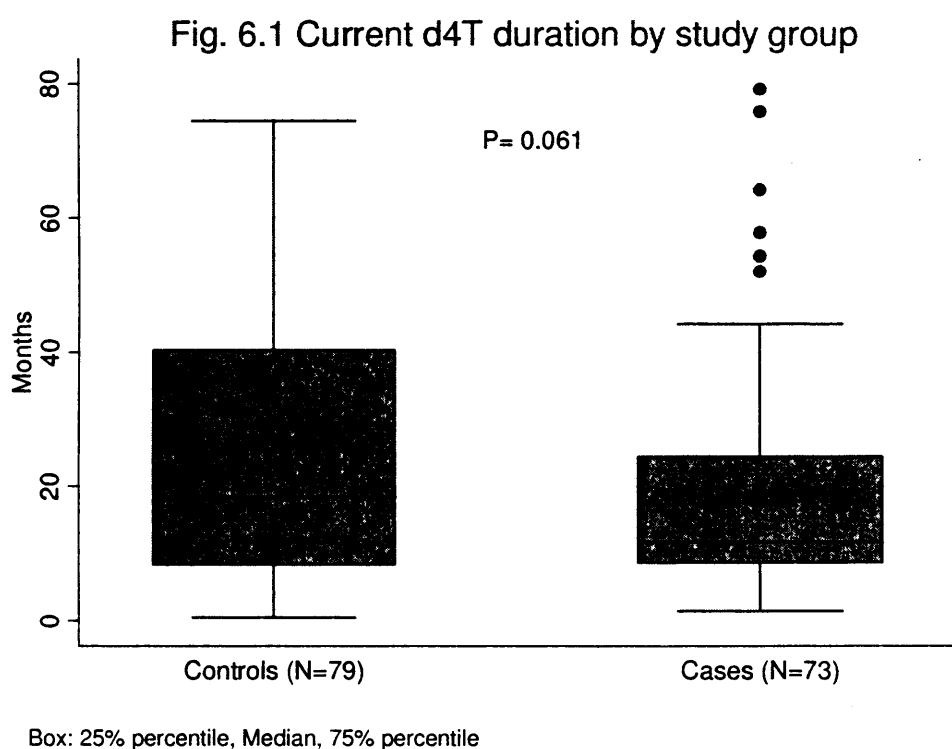
Table 6.8 Laboratory tests results at the time of the event

Variable	Cases			Controls			P
	N	Median	IQR* (Range)	N	Median	IQR* (Range)	
pH	78	7.32	7.25 - 7.40 (6.88 - 7.47)				NA**
Lactate (mmol/l)	110	6.75	5.54 - 8.1 (2.4 - 20.3)	62	1.4	1.1 - 1.88 (0.64 - 3.4)	NA**
Bicarbonate (mmol/l)	98	18	13.1 - 21.6 (2.2 - 29)	30	25	23 - 26 (21 - 31)	NA**
WCC (x10 ¹² /l)	101	6.7	4.9 - 9.07 (0.4 - 22.7)	192	5.5	4.5 - 7.08 (1.6 - 12.2)	0.01
Neutrophils (x10 ⁹ /l)	94	3.95	2.5 - 6.19 (0.12 -20)	190	2.99	2.2 - 4.02 (0.7 - 9.3)	< 0.001
Hb (gr/dl)	101	13.2	10.5 - 14.4 (4 - 17.8)	194	13.5	11.5 - 14.9 (5.9 - 18.5)	0.016

* IQR= interquartile range

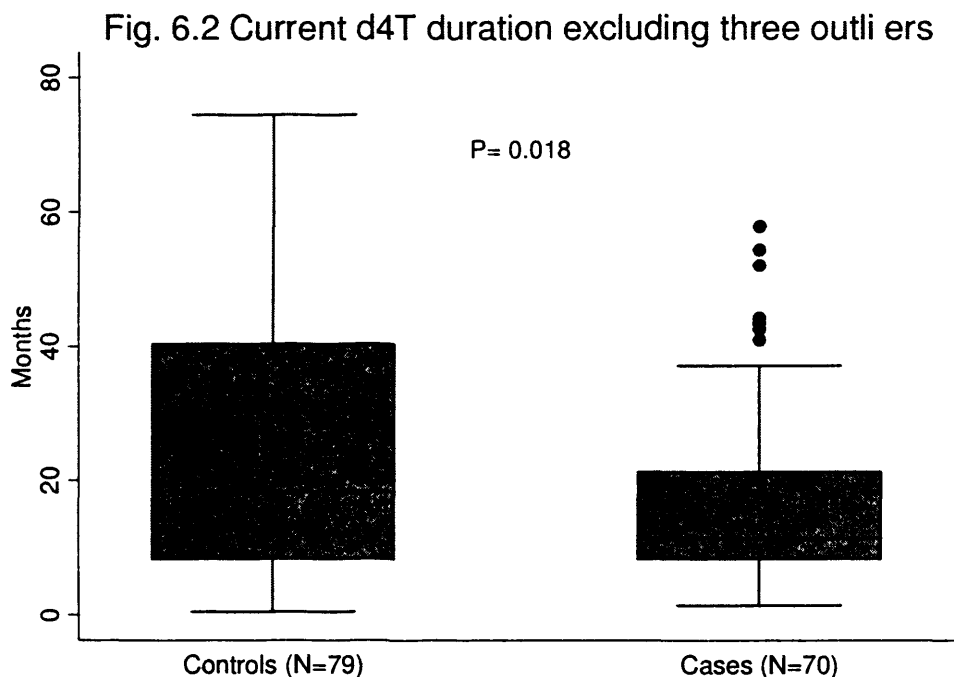
** NA= non applicable

Three of these subjects had additional factors which may have affected their risk of developing HL/LA. One female patient was an IVDU with HIV/HCV co-infection who after many years on d4T/ddI started therapy with Ribavirin and Pegylated Interferon α (Peg Inf). She was reluctant to switch ART, and after 2 months exposure to Rib/Peg Inf she developed a severe and fatal episode of LA. The second male patient had been stable on d4T/ddI for a few years. He had an episode of acute intoxication, assumed to be due to scopolamine, and was found unconscious at his home. Toxicological screening was not performed but high alcohol consumption was a possibility considered. The patient survived the episode of HL/LA. The third patient was also a male and the only case of HL among these three. He was 70 years old and had a diagnosis of Parkinson's disease on treatment with co-careldopa for over two years at the time of the event. Parkinson's disease has been described as a mitochondrial disease. This patient also had Parkinson's disease-related myopathy at the time of the event.

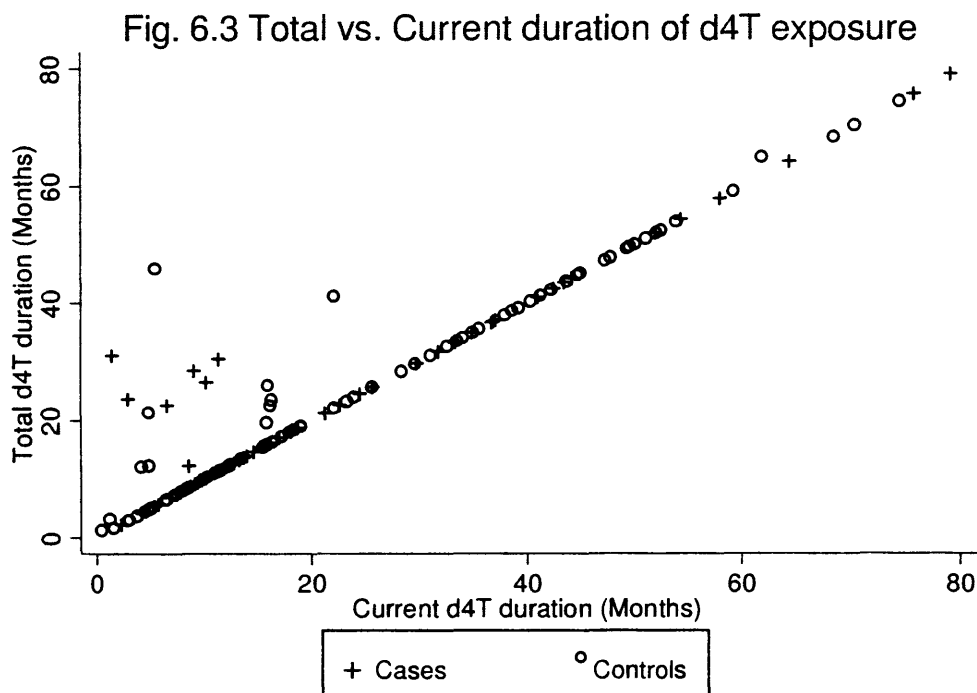


Excluding these three outliers the difference in current d4T duration between cases and controls becomes significant (11.46 months [IQR= 8.42 – 21.28])

versus 18.42 months [IQR= 8.32 – 40.33] Mann-Whitney test $P= 0.018$) (Fig. 6.2). Interestingly, as two of these patients were on ddl as well at the time of the event, they may also be excluded from the analysis for ddl current duration. After excluding these two patients, the median ddl duration for cases ($N= 62$) was 11.56 months (IQR= 8.49 – 21.28) and it was 15.89 months (IQR= 7.04 – 34.57) for controls ($N= 55$) ($P= 0.180$).



Of interest, most of those patients who were receiving d4T-based combinations at the time of the event had not had a previous exposure, i.e. course of the drug. As shown in Fig. 6.3, only 10 cases (9.1 %) and 12 controls (5.5 %) of those patients on d4T at the time of being included in the study had been previously exposed to d4T and so the duration of their total exposure to the drug is longer than the duration of their current exposure.

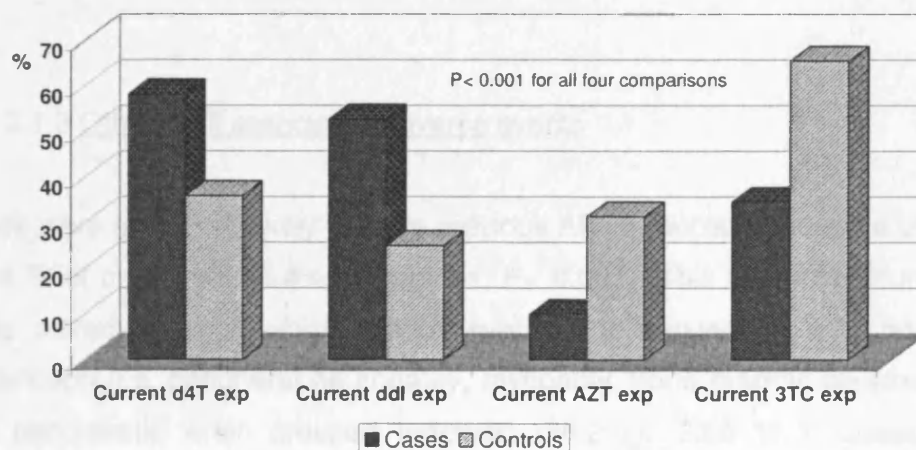


Cases were significantly more likely to be receiving d4T or ddl at the time of the event whereas controls were more likely to be receiving AZT or 3TC at the time of being included in the study (Fig 6.4). The majority of cases who were taking d4T at the time of the event were simultaneously receiving ddl (64 %) whereas most of controls who were on d4T at the time of being included in the study were taking it in combination with 3TC (61 %). Similarly, 73 % of cases on ddl at the time of the event were on combination regimens which included d4T. Interestingly, almost half (49 %) of controls receiving ddl at the time of being selected were taking it in combination with d4T.

Most participants were receiving combination ART at the time of being included in the study. Therefore, almost all participants were taking at least two NRTIs at the time of their inclusion. Seven cases stopped all ART drugs a few days before the diagnosis of the event was made (median= 10.5 days; range= 1 – 15). All these seven patients stopped medication because of symptoms associated with the event. Therefore, these seven cases were

included in the study as currently exposed to ART. One case was receiving a combination which included only one NRTIs (emtricitabine –FTC-) together with efavirenz and atazanavir at the time of the event. Another case was receiving a combination of five ART drugs which included d4T, ddl, 3TC and ABC. An additional case was exposed to d4T/Efv/Nfv for 8.5 months but stopped his d4T seven months before the event (reason not recorded in his clinical notes). That patient remained on efavirenz and nelfinavir until the event was diagnosed. No AIDS defining conditions were reported at the time of the event for that patient. Finally, there was a case who had been off ART for over two years by the time of the event. That final male patient had been exposed to d4T/3TC/Nfv for 10.2 months before stopping all ART medication two years before the event. Patient's choice was the reason for stopping ART.

Fig. 6.4 Current NRTI exposure



Cases were more likely to be exposed to d4T and ddl simultaneously (d4T/ddl) than controls. A total of 47 cases (43 %) were receiving d4T/ddl at the time of the event whereas the number of controls receiving such a combination was 27 (12.3 %) ($P < 0.001$). On the contrary, controls were more likely of being currently exposed to a combination of AZT and 3TC (29 %) than cases (9 %) ($P < 0.001$). Interestingly, similar proportions of cases and controls were taking d4T/3TC at the time of being recruited (17 % and 22 % respectively; $P = 0.321$) (Table 6.6).

Table 6.6 NRTI combinations at the time of inclusion

NRTI Combination	Cases		Controls		P
	N	%	N	%	
d4T / ddl	47	42.7	27	12.3	< 0.001
d4T / 3TC	19	17.3	48	21.8	0.228
d4T / ABC	7	6.4	4	1.8	0.085
ddl / 3TC	10	9.1	12	5.5	0.202
ddl / AZT	1	0.9	7	3.2	0.174
ddl / ABC	4	3.6	8	3.6	1.00
ddl / TDF	10	9.1	7	3.2	0.011
AZT / 3TC	10	9.1	63	28.6	< 0.001

6.2.1.3 Other ART associated adverse events

Cases were also more likely to have previous ART-associated adverse events (76.4 % of cases vs. 56.4% of controls; $P < 0.001$). This was also found for those adverse events which are believed to be caused by mitochondrial dysfunction (i.e. peripheral neuropathy, myopathy, bone marrow suppression and pancreatitis when grouped together) (38.2 vs. 23.6 % in cases and controls respectively; $P = 0.008$) and when analysed alone; previous episodes of PN (27.3 % vs. 6.8 %; $P < 0.001$), pancreatitis (12.7 % vs. 0.9 %; $P < 0.001$) and myopathy (6.4 % vs. 1.4 %; $P = 0.018$). However, there was no significant difference between the proportion of cases (3.7 %) and controls (0.9 %) who had previous episodes of bone marrow suppression ($P = 0.096$). As described in chapter 5, for this analysis, all episodes described before the last regular appointment with each patient's regular doctor was considered as previous and all episodes diagnosed from that last appointment to the date of the event were assumed as current.

6.2.1.4 Clinical and biochemical presentation of the event

Clinical data were not included in the study case definitions but symptoms at the time of diagnosis of the event (HL/LA) for cases or at the time of being included in the study for controls were recorded. Interestingly, 10 cases (9.1 %) were completely asymptomatic at the time of the event. All 10 cases were HL. So, 16.4 % of those individuals with severe hyperlactataemia (N= 61) were asymptomatic at the time of diagnosis. The median blood lactate of these 10 asymptomatic cases was 5.8 mmol/l (IQR= 5.4 – 6.8) but two of them had blood lactate above 7 mmol/l.

Table 6.7 shows the frequency of observed symptoms among both cases and controls. There were 107 cases in whom symptoms at the time of diagnosis were recorded. Sixty nine out of these 107 cases (64.5 %) had gastrointestinal symptoms (i.e. abdominal pain, nausea and/or vomiting). Besides, 61/107 of the cases (57 %) had constitutional symptoms (i.e. anorexia, fatigue, weight loss and/or weakness). Central nervous system (CNS) impairment, mostly impairment of consciousness, was reported in five cases (4.7 %) but it must be highlighted that all these five cases had LA indicating that 10.9 % of those patients included in the study with LA had CNS impairment at the time of diagnosis. Similarly, 17 cases had dyspnoea at the time of diagnosis and 11 out of these 17 dyspnoeic patients were cases of LA.

The median blood lactate among cases at the time of the event of HL/LA was 6.75 mmol/l (IQR= 5.54 – 8.1) whereas for the 62 controls in whom that parameter was assessed it was 1.4 mmol/l (IQR= 1.1 – 1.88) (Table 6.8). Some centres include blood lactate in their routine screening procedures for patients in follow-up. Four (6.5%) of the controls had levels higher than 2.2 mmol/l. The values ranged between 2.5 and 3.4 mmol/l. All four were asymptomatic at the time of their inclusion in the study.

Table 6.7 Symptoms reported at the time of the event or inclusion in the study

	Cases		Controls	
	N	%	N	%
Nausea	51	47.7	4	1.8
Vomiting	46	43.0	1	0.5
Abdominal pain	43	40.2	4	1.8
Anorexia	31	29.0	0	0.0
Fatigue	37	29.0	7	3.2
Weight loss	30	27.8	9	4.1
Weakness	27	25.2	7	3.2
Dyspnoea	17	15.9	0	0.0
Tachypnoea	9	8.4	1	0.5
CNS impairment	5	4.7	0	0.0
Jaundice	3	2.8	1	0.5
Other	34	31.8	14	6.4
Asymptomatic	10	9.1	182	82.7

Ninety eight cases had blood bicarbonate assessed at the time of the event (49 LA and 49 HL). The median blood bicarbonate for this group of cases was 18 mmol/l (IQR= 13.1 – 21.6). Data on bicarbonate were available from 30 control patients who all showed normal levels at the time of being included in the study. 68 out of the 98 cases (69 %) with blood bicarbonate results available had abnormal values (< 20 mmol/l) at the time of the event. Interestingly, 8 out of these 68 patients with abnormal bicarbonate (11.8 %) had a blood lactate lower than 5 mmol/l. All were acidotic at the time of their inclusion. On the other hand, 20 cases of HL (with no abnormal pH by definition) had low blood bicarbonate levels at the time of the event ranging from 12.2 to 19.7 mmol/l. Arterial blood pH was assessed in 78 cases (49 LA and 29 HL). The median blood pH for those cases was 7.32 (IQR= 7.25 – 7.40).

Cases were more likely to have abnormal haematological laboratory results at the time of the event compared with controls (Table 6.8). The median white blood cell count (WBCC) for cases (N= 101) was 6.7 cell x 10¹²/l (IQR= 4.9 –

9.1) and for controls (N= 192) it was $5.5 \text{ cell} \times 10^{12}/\text{l}$ (IQR= 4.5 – 7.1) ($P < 0.001$). Of note, only 24 cases had an abnormal WBCC at the time of the event: 17 cases had WBCC $> 10 \text{ cell} \times 10^{12}/\text{l}$ (median= $13.4 \text{ cell} \times 10^{12}/\text{l}$; IQR= 12.3 – 15.8) and seven cases had WBCC < 3 (median= $2.0 \text{ cell} \times 10^{12}/\text{l}$; IQR= 1.6 – 2.3). The median neutrophil count for cases was $3.95 \text{ cell} \times 10^9/\text{l}$ (IQR= 2.5 – 6.2) for cases and 2.99 (IQR= 2.2 – 4.0) for controls ($P < 0.001$). There were 25 cases with abnormal neutrophil counts: 13 with neutrophils $> 7.5 \text{ cell} \times 10^9/\text{l}$ (median= $10.9 \text{ cell} \times 10^9/\text{l}$; IQR= 8.4 – 13.1) and 12 with neutrophils count $< 2 \text{ cell} \times 10^9/\text{l}$ (median= $1.3 \text{ cell} \times 10^9/\text{l}$; IQR= 1.0 – 1.7). As mentioned before, the median CD4 count at the time of the event was 248 cell/ μl (IQR= 136.5 – 365) for cases compared to 400 cell/ μl (IQR= 220 – 551.5) for controls ($P < 0.001$).

There was a significant amount of missing data on laboratory results, particularly among controls. In general, more investigations were performed in cases than in controls at the time of being included in the study. That may be because most controls were attending regular follow-up visits. However, from the available data cases were more likely to have abnormal liver function tests ($P < 0.001$), as well as abnormal albumin ($P < 0.001$), amylase ($P = 0.034$), glucose ($P < 0.001$), creatinine ($P = 0.021$) and urea ($P < 0.001$) at the time of the event when compared to controls (Table 6.9). Interestingly, among the 30 cases with abnormal blood creatinine values at the time of the event 11 had levels below the reference normal range for the respective laboratory whereas 19 of the cases with abnormal creatinine level (19 % of the cases with blood creatinine results at the time of the event) had values above the upper limit of the respective normal range. Furthermore, the actual level of creatinine among these 19 patients ranged between 1.2 and 19.2 times over the upper limit of normal range and only two of these cases had levels > 4 times the upper normal value. The first of these two patients had creatinine levels above 7 times higher than the upper limit of normal. It was a male patient who had previous diagnosis of diabetes mellitus on treatment with insulin. In addition, his blood urea level was more than three times higher than the upper limit of normal. He had also developed renal calculi during previous exposure to

indinavir. The other case with very abnormal creatinine level was a male patient with creatinine values 19 times higher than the upper limit of normal. That patient was on salvage combination therapy which included ddI in combination with TDF plus ABC, 3TC and boosted APV.

Chronic hepatitis B virus (HBV) infection was defined as the presence of a positive HBsAg result for longer than six months. A total of eight out of 85 tested cases (9.4 %) were HBsAg positive whereas 10 out of 159 tested controls (6.3 %) were identified as chronically HBV infected ($P = 0.644$). Likewise, 22 out of 80 tested cases (27.5 %) and 26/159 controls (16.4 %) were identified as chronically hepatitis C infected (AntiHCV positive) ($P = 0.103$).

However, because of the missing data and the matched analysis strategy used it was difficult to fit laboratory variables into a regression model. The matched analysis implies that a missing value for a control means losing its respective case for the analysis as well. In addition, laboratory markers may be considered as factors that are a consequence of the event rather than potential risk factors. Therefore, laboratory variables other than those assessing HIV disease severity were not included in the further analysis.

Table 6.9 Frequency of abnormal laboratory results

	Cases			Controls			P
	Abnormal*	Tested**	%	Abnormal*	Tested**	%	
ALP	52	99	52.5	30	194	15.5	< 0.001
ALT	62	93	66.7	43	183	23.1	< 0.001
AST	45	59	76.3	16	116	13.8	< 0.001
Albumin	24	81	29.6	5	122	4.1	< 0.001
Amylase	24	76	31.6	11	109	10.1	0.034
Glucose	41	86	47.7	12	132	9.1	< 0.001
Creatinine	30	102	29.4	31	180	17.2	0.021
Urea	29	79	36.7	7	120	5.8	< 0.001

* Number of patients with abnormal results

** Number of patients tested

Abdominal ultrasound scans were performed at study entry in 51 participants (34 cases and 17 controls). Sixteen of the cases (47.1 %) had evidence suggestive of hepatic steatosis and eight controls (47.1 %) also showed similar changes. Five cases (14.7 %) had hepatomegaly with no evidence of fatty infiltration or inflammation. Two cases had images suggestive of cirrhosis and 11 cases (32.4%) showed normal appearance of their liver. In eight controls liver ultrasound scanning was reported as normal. The final control had hepatomegaly as the major abnormality found. It is important to mention that this may be a biased sub-set as abdominal ultrasound is not a routine test in any of the participant centres and therefore it may be performed only in those patients with clinical or biochemical abnormalities.

6.2.1.5 Mitochondrial function

Mitochondrial function tests were performed in only three cases. They were all males aged 69, 47 and 41 years. Two had mitochondrial studies performed in skeletal muscle cells and in PBMC and one in muscle cells but this was the only case in whom mitochondrial morphology was assessed by electron microscopy. Table 6.10 shows a summary of mitochondrial function tests carried out in these three cases. Of note, all three patients had normal function of mitochondrial respiratory chain complex II in at least one cell type studied. Nevertheless, case 1 had normal complex II activity in muscle cells but a mild reduction (73% of the activity measured in healthy controls) in PBMC. On the contrary, case 2 had normal complex II activity in PBMC but a significant reduction in complex II activity (51 % of controls' activity) in muscle cells. Complexes I and III activities were reduced in the muscle cells of all cases. The ratio mt : nDNA was significantly reduced in PBMC of those cases in whom PBMC were examined (cases 1 and 2) (Lopez *et al.* 2002). However, case 2 with mtDNA depletion in PBMC had normal mtDNA content in muscle cells. Case 2 was receiving treatment with ddI/TDF/Efavirenz at the time of the event and had been diagnosed with renal impairment (Creatinine in blood 2.3

times over the upper limit of the normal at the time of the event). The patient with mtDNA depletion in both muscle and peripheral blood mononuclear cells (case 1) had previous diagnosis of Parkinson's disease and concurrent diagnosis of myopathy at the time of the event and was on d4T/ddI/TDF. None of these two patients were acidotic and their blood lactate level at the time of the event were 10.4 and 5.2 mmol/l respectively.

Table 6.10 Mitochondrial function tests

Cases	CI*	CII	CIII	CIV	mt : nDNA**
1 Male (69 years old)					
Muscle	Reduced	Normal	Reduced	Reduced	Reduced
PBMC	ND***	Reduced	Reduced	Reduced	Reduced
2 Male (41 years old)					
Muscle	Reduced	Reduced	Reduced	Normal	Normal
PBMC	ND***	Normal	Normal	Normal	Reduced
3 Male (47 years old)					
Muscle	Reduced	Normal	Reduced	Reduced	Normal

* CI-IV= Respiratory chain complexes I to IV

** mt : nDNA = mitochondrial/nuclear DNA ratio

*** ND= Not done

The remaining case (case 3) had significant reduction of all mtDNA encoded respiratory chain complexes but normal complex II activity. Surprisingly, the patient had normal mtDNA content in muscle cells. However, ultra-structural studies showed abnormal mitochondria with giant forms and abnormal matrix content. The patient was receiving treatment with AZT/ddI at the time of the event of LA (pH= 7.13; bicarbonate= 5.7 mmol/l; lactate= 6.4 mmol/l) (Miller *et al.* 2003).

6.2.2 Risk factors for HL/LA: Univariate analysis

Table 6.11 shows a summary of the univariate analysis performed to identify risk factors for HL/LA. Older patients were at higher risk of developing HL/LA (odds ratio (OR) 1.03 per year (95% CI= 1.01 – 1.06) and when age was measured as a categorical variable patients older than 40 years (closest entire

value to the median age for the study population) were 87 % more likely to develop HL/LA (OR= 1.87; 95% CI= 1.13 – 3.09) than younger patients.

Female patients were about 3 times more likely to develop HL/LA than male participants (OR= 3.27; 95% CI= 1.78 – 6.03) and as previously described cases were more likely to have been infected by heterosexual exposure (OR= 3.42; 95% CI= 1.75 – 6.69).

Although it is likely that only a particular group of patients, such as those with symptoms likely to be related to mitochondrial dysfunction would be expected to have had previous blood lactate and perhaps amylase measurements, and therefore any analysis performed using such variable may be biased, patients with previous abnormal lactate and amylase results (at any time before the last regular follow-up visit the patient had before the event) were at significantly higher risk of developing HL/LA (OR= 4.74; 95% CI= 1.98 – 11.30 and (OR= 5.11; 95% CI= 1.99 – 13.10 respectively).

Patients with more advanced HIV disease at the time of being included in the study were at significantly higher risk of developing the study outcome. Those individuals with concurrent OI and CD4 < 200 were more than twice and three times likely to develop HL/LA respectively compared to those who did not have concurrent OI at the time of their inclusion in the study (OR= 2.44; 95% CI= 1.19 – 5.03 and OR= 3.63; 95% CI= 1.94 – 6.82 respectively).

Those patients with concurrent ADR likely to be due to mitochondrial dysfunction were at a particularly higher risk of developing HL/LA when compared with those who did not (OR= 5.87; 95% CI= 3.14 – 10.98). Nevertheless, it is impossible to elucidate whether or not the concurrent mitochondrial toxicity may be part of the clinical presentation of an episode of HL/LA. In addition, even if the episode of HL/LA were an independent condition that is likely to be the most severe mitochondrial toxicity associated with ART and other concurrent (and even recent previous) mitochondrial pathologies may be steps in the pathogenic pathway to extreme

mitochondrial dysfunction. Therefore, it was considered sensible to exclude concurrent mitochondrial dysfunctions from the multivariate analysis. Also dates of previous ADR were not recorded and so it was not possible to know how close in time previous ADR and the study event were to each other. It was therefore decided to exclude previous ADR from the multivariate model.

Patients receiving d4T based ART combinations were more than twice as likely to develop HL/LA compared to those who were taking combinations that did not include it. In the same way, patients taking ART combinations which included ddI were at much higher risk compared to those who were not taking non-ddI containing combinations. On the contrary, in the univariate analysis 3TC and AZT exposure were associated with “protective” effects for HL/LA. Patients on 3TC based ART combinations were 73 % less likely to have HL/LA compared to those who were not taking such a drug as part of their ART and patients on AZT based ART combinations were 75 % less likely to develop HL/LA than those not taking that.

Patients taking combination ART which include TDF were also at higher risk for HL/LA (OR= 3.16; 95% CI= 1.17 – 8.50). Ten of the 15 cases (67 %) who were taking TDF at the time of the event were doing so in combination with ddI. Interestingly, four of those cases taking ddI/TDF were also taking d4T as part of their ART combination. In addition, four of the remaining cases on TDF at the time of the event were taking that in combination with 3TC and the final one was taking TDF in combination with AZT.

Univariate analyses were also performed examining the duration of the exposure of each ART drug as the independent variable. From such analyses longer exposures to d4T were associated with a “protective effect” for HL/LA (Table 6.12). The OR associated with the duration of current d4T exposure was 0.97 (95% CI= 0.94 - 1.00) per month and the OR associated with current d4T exposures longer than 15 months (median of the current d4T duration of the entire study population) was 0.27 (95% CI= 0.11 – 0.68).

Table 6.11 Univariate analysis : Risk factors for HL/LA

Variable	CASES	CONTROLS	OR	95 % CI	P
	Freq/Total (%)	Freq/Total (%)			
Demographics					
Age (per year)			1.03	1.01 - 1.06	0.011
Gender					
Male	70/110 (63.6)	180/220 (81.8)	1		< 0.001
Female	40/110 (36.4)	40/220 (18.2)	3.27	1.78 - 6.03	
Mode of infection					
Homosexual	41/100 (41.0)	125/199 (62.8)	1		0.001
Heterosexual	40/100 (40.0)	47/199 (23.6)	3.42	1.75 - 6.69	
IVDU	14/100 (14.0)	23/199 (11.6)	2.44	1.04 - 5.70	
Blood Transfusion	5/100 (5.0)	4/199 (2.0)	3.74	0.95 - 14.74	
HIV history					
Previous AIDS diagnosis	59/110 (53.6)	82/220 (37.3)	2.08	1.27 - 3.40	0.004
Nadir CD4 (< 200)	69/91 (75.8)	101/188 (53.7)	4.38	2.08 - 9.22	< 0.001
Previous ART-associated ADR	84/110 (76.4)	124/220 (56.4)	3.00	1.67 - 5.40	< 0.001
Previous MT adv effects (ART)	42/110 (38.2)	52/220 (23.6)	1.95	1.19 - 3.19	0.008
Past medical history					
Abnormal lactate	24/110 (21.8)	17/220 (7.7)	4.74	1.98 - 11.30	< 0.001
Abnormal LFT	56/110 (50.9)	65/220 (29.6)	3.95	2.11 - 7.41	< 0.001
Abnormal amylase	17/110 (15.5)	9/220 (4.1)	5.11	1.99 - 13.10	0.001
Concurrent medical events					
Opportunistic infections	19/110 (17.3)	18/220 (5.2)	2.44	1.19 - 5.03	0.015
ART-associated adverse effect	73/110 (66.4)	83/220 (37.9)	3.58	2.13 - 6.01	< 0.001
ART-associated MT adv effect	43/110 (39.1)	20/220 (9.1)	5.87	3.14 - 10.98	< 0.001
CD4 (< 200)	45/100 (45.0)	49/212 (23.1)	3.63	1.94 - 6.82	< 0.001
ART exposure					
Current d4T exp	73/110 (66.4)	79/220 (35.9)	3.67	2.20 - 6.14	< 0.001
Current ddl exp	64/110 (58.2)	55/220 (25.0)	5.54	3.04 - 10.10	< 0.001
Curent 3TC exp	40/110 (36.4)	144/220 (65.5)	0.27	0.16 - 0.46	< 0.001
Current AZT exp	11/110 (10)	69/220 (31.4)	0.25	0.12 - 0.50	< 0.001
Current TDF exp	15/110 (13.6)	15/220 (6.8)	3.16	1.17 - 8.5	0.023
Current Hyd exp	5/110 (4.6)	1/220 (0.5)	9.99	1.17 - 85.59	0.036
Current d4T/ddl exp	47/110 (42.7)	27/220 (12.3)	8.70	4.05 - 18.65	< 0.001
Current AZT/3TC exp	10/110 (9.1)	63/220 (28.6)	0.25	0.12 - 0.51	< 0.001
Current ddl/TDF exp	10/110 (9.1)	7/220 (3.2)	4.85	1.29 - 18.20	0.019

As was described before (see section 6.2.1) there were three cases who may be considered as outliers: patients who had received d4T containing regimes for periods much longer than the majority of the included cases who had been exposed to d4T. If these three cases are excluded from the analysis, the effect

of d4T duration is somehow more evident suggesting that the risk of developing HL/LA among those patients exposed to d4T may decrease by 4 % each month (OR= 0.96; 95% CI= 0.92 – 0.99) per year. Conversely, exclusion of potential outliers from the analysis on duration of ddI exposure did not modify the effect of duration of such exposure on the risk of developing HL/LA. Nevertheless, it is important to highlight that all OR in that analysis were very close to one, even when the significance tests are below the previously specified significance level, so they should be interpreted cautiously.

Finally, as almost all patients were receiving combination therapy at the time of being included in the study and therefore most were taking at least two NRTIs simultaneously, univariate analyses were performed to assess the effect of the most frequently used NRTIs combinations. As mentioned before, most patients were taking at least two NRTIs in combination at the time of their inclusion in the study. In addition, NRTIs combinations are not randomly allocated and therefore it is difficult to assess the effect of a individual drug as that may be prescribed very often with one other specific NRTIs.

As expected, those patients who were receiving d4T/ddI combination therapy were about eight times more likely to develop HL/LA (OR= 8.70; 95% CI= 4.05 – 18.65) when compared to patients on different combinations. Conversely, exposure to AZT/3TC combination at the time of being included in the study was a protective factor for the study outcome (OR= 0.25; 95% CI= 0.12 – 0.51).

In addition, patients who were receiving combinations which included ddI and TDF had more than four times the risk of developing HL/LA than patients who were not receiving these two drugs in combination (OR= 4.85; 95% CI= 1.29 – 18.20) (Table 6.11).

Table 6.12 Univariate analysis : Effect of d4T and ddl duaration

Variable	Cases			Controls			OR	95% CI
	N	Median	IQR (Range)	N	Median	IQR* (Range)		
Current d4T duration	73	11.74	8.6-24.5 (1.5-79.1)	79	18.42	8.3-40.3 (0.5-74.4)	0.97*	0.94 - 1.00
Current d4T duration exc outliers**	70	11.46	8.4-21.3 (1.5-57.8)	79	18.42	8.3-40.3 (0.5-74.4)	0.96*	0.92 - 0.99
Current d4T duration > 15 months	47			29			0.27	0.11 - 0.68
Current ddl duration	64	11.68	8.6-23.6 (1.2-115.2)	55	15.89	7.0-34.6 (0.2-83.4)	0.99*	0.96 -1.02
Current ddl duration exc outliers**	62	11.56	8.5-21.3 (1.2-54.3)	55	15.89	7.0-34.6 (0.2-83.4)	0.96*	0.91 - 1.00

* Per month

** Three patients on d4T and two on ddl

6.2.3 Risk factors for HL/LA: Multivariate analysis

Table 6.13 shows the multivariate model developed to identify risk factors for HL/LA. As was mentioned above, a few variables which were found to be significantly associated with the study outcome in the univariate analysis were not included in the model. These variables were previous AIDS diagnosis, nadir CD4 and laboratory results other than lymphocyte CD4 count at the time of inclusion in the study.

After adjusting for other factors, both age and female gender remained strongly associated with the study outcome. Older patients were at higher risk of developing HL/LA and the risk associated with age increases about 4 % per year. In addition, the effect of gender on the likelihood of developing HL/LA remained quite strong in the multivariate model. Female patients were more than four times more likely to develop the study outcome than male participants.

Table 6.13 Multivariate model : Risk factors for HL/LA

Variable	Univariate			Multivariate		
	OR	P	95 % CI	OR	P	95 % CI
Age (per year)	1.03	0.011	1.01 - 1.06	1.04	0.036	1.00 - 1.08
Gender (female)	3.27	< 0.001	1.78 - 6.03	4.75	0.001	1.96 - 11.53
CD4 (< 200)	3.63	< 0.001	1.94 - 6.82	3.44	0.001	1.64 - 7.22
Current d4T exp	3.67	< 0.001	2.20 - 6.14	4.55	< 0.001	2.54 - 9.18
Current ddl exp	5.54	< 0.001	3.04 - 10.10	4.92	< 0.001	2.30 - 10.51

Similarly, patients with more advanced HIV-induced immunosuppression were also at higher risk of developing HL/LA according to the multivariate model. Participants with CD4 count lower than 200 cell/ μ l at the time of their inclusion in the study were more than three times more likely to develop HL/LA than those patients with current higher CD4 count.

Exposures to d4T and ddI were all strongly associated with the study outcome. The risks of developing HL/LA for those currently exposed to d4T or ddI were more than four times as high as patients not receiving these drugs. In contrast, exposures to either AZT or 3TC, examined individually rather than in combination after adjusting for other factors, lost the “protective” effect for HL/LA seen in the univariate analysis.

All patients included in the study had been exposed to combination ART, even if they were not taking ART at the time of inclusion in the study: one case and 16 controls were off therapy at the time of inclusion. Therefore, most of the study participants were taking at least two NRTIs simultaneously. As mentioned before, specific combinations were frequently seen in study participants (e.g. d4T/ddI, AZT/3TC, ddI/TDF) and therefore it is difficult to assess the effect of specific drugs on the study outcome when they are very often used in association with other specific drugs. As a result, it was considered appropriate to build up a multivariate model based on drug combinations rather than single drug exposures. Table 6.14 shows the resulting model.

Table 6.14 Multivariate model based on NRTI combinations

	Univariate			Multivariate		
	OR	P	95 % CI	OR	P	95 % CI
Age (per year)	1.03	0.011	1.01 - 1.06	1.04	0.015	1.01 - 1.08
Gender (female)	3.27	< 0.001	1.78 - 6.03	3.57	0.003	1.53 - 8.35
CD4 (< 200)	3.63	< 0.001	1.94 - 6.82	3.64	< 0.001	1.78 - 7.48
d4T/ddI exp	8.70	< 0.001	4.05 - 18.65	6.42	< 0.001	2.60 - 15.83
AZT/3TC exp	0.25	< 0.001	0.12 - 0.51	0.35	0.028	0.14 - 0.89
ddI/TDF exp	4.85	0.019	1.29 - 18.20			

The model described in table 6.14 shows little difference from the model presented earlier which considered individual NRTIs exposures. The effect of age, gender and current CD4 count (< 200 cell/μl) remained almost exactly the same when adjusted for NRTIs combinations rather than single drug

exposures. However, the effect of 3TC when combined with AZT remained significantly associated with the study outcome. In fact, those patients exposed to AZT/3TC based combinations were 65 % less likely to develop HL/LA.

As expected, the combination of d4T and ddI showed a strong effect on HL/LA. Patients receiving d4T/ddI based combinations were much more likely to develop the study outcome (OR= 6.42; 95 % CI= 2.60 – 15.83). However, because of the relatively wide confidence intervals it is difficult to assess the actual magnitude of the increased attributable risk.

6.2.4 LA and HL as independent outcomes

As described before, cases of LA and cases of HL were combined in a single outcome for the study analysis. Nevertheless, a secondary objective of the study was to assess similarities and differences between risk factors associated with these two entities. Separate analyses were therefore performed considering each outcome individually. Conditional logistic regression methods were used to take into consideration matching variables.

Table 6.15 shows the distribution of demographic, HIV-related and ART exposure variables by separate outcomes. Considering both together, cases of HL and LA were significantly older than controls (see section 6.2.1). Similarly, cases of HL were also significantly older than controls, with a median age at the time of the event of 44.8 years (IQR= 37.6 – 54.7) whereas the median age of controls at the time of being included in the study was 40 years (IQR= 35.0 – 47.1) ($P= 0.001$). However, the median age of cases of LA (median= 41.1 years; IQR= 35.1 – 48.8) was not significantly different from the controls median age ($P= 0.806$).

Table 6.15 Cases of LA and HL: descriptive analysis

		LA (N= 49*)		HL (N=61*)	
		N° Events	%	N° Events	%
Age					
	< 40 years	21	42.9	19	13.2
	> 40 years	28	57.1	42	68.9
Gender					
	Male	26	53.1	44	72.1
	Female	23	46.9	17	27.9
Ethnicity		(N= 36)		(N= 50)	
	White all	21	58.3	29	58.0
	Black all	12	33.3	10	20.0
	Asian	0	0.0	5	10.0
	Hispanic	3	8.3	6	12.0
	Other	0	0.0	0	0.0
HIV history					
	AIDS	30	61.2	29	47.5
	Concurrent OI	8	16.3	11	18.0
	Concurrent mt AE	27	55.1	16	26.2
Current NRTI exposure					
	d4T	28	57.1	45	73.8
	ddl	25	51.0	39	63.9
	3TC	24	49.0	16	26.2
	AZT	5	10.2	6	9.8
	TDF	8	16.3	7	11.5
	d4T/ddl	15	30.6	32	52.5
	AZT/3TC	4	8.2	6	9.8
Outcome					
	Fatal outcome	16	32.7	3	4.9

* unless a different figure is indicated

The gender distribution was also somewhat different between cases of LA and HL. Twenty three cases of LA (46.9 %) were female while among controls only 40 individuals (18.2 %) were female ($P= 0.001$). The proportion of female cases among those with HL diagnosis (17/61; 27.9 %) was also higher than the proportion described among controls (40/220; 18.2 %), but this difference did not reach statistical significance ($P= 0.087$).

Both patients with LA and HL had CD4 lymphocyte counts significantly lower than controls at the time of the event. The median CD4 count of cases with LA at the time of the event was 172 cell/ μ l (IQR= 100 – 360) and the median CD4 count for cases of HL was 255 cell/ μ l (IQR= 150 – 390) while the current CD4 count of controls was 400 cell/ μ l (IQR= 220 – 551.5). Both differences were significant ($P < 0.001$ and $P = 0.002$ respectively).

Cases of LA as well as cases of HL were more likely to be exposed to d4T (53.1 % and 67.2 % respectively) or ddl (49 % and 60.6 % respectively) at the time of the event than controls (35.9 % for d4T and 25 % for ddl) (Table 6.14). The proportion of cases of LA and HL when considered as separate outcomes who were on d4T at the time of the event were significantly different from the same figure among controls ($P = 0.046$ and $P = 0.001$ respectively) which is similar to what was found analysing ddl exposure ($P < 0.001$ for both outcomes).

Patients with both LA and HL diagnoses combined as single outcomes were less likely than controls to receive AZT and 3TC at the time of being included in the study. Ten percent of the cases of LA and HL were receiving AZT compared with 31.4 % of controls who were on AZT at the time of being included ($P = 0.009$ and $P = 0.004$ respectively). In addition, 49 % of cases of LA were on 3TC whereas the proportion of cases of HL on that drug was 26.2 %. Both figures were significantly different from the 65.5 % of controls who were on 3TC at the time of their inclusion ($P = 0.043$ and $P < 0.001$ respectively).

All cases of LA were symptomatic at the time of the event whereas 10 of the 61 cases of HL (16.4 %) were described as symptom free at the time of diagnosis (Table 6.16). Nevertheless, the distribution of symptoms among those cases of HL who had clinical manifestations at the time of the event was very similar to what was found among cases with LA. The most frequently

described symptoms such as gastro-intestinal or constitutional symptoms (e.g. fatigue, weight loss, etc) were very similar between cases of LA and HL. Nonetheless, dyspnoea and tachypnoea were more frequently seen in patients with LA (24 % and 13 % respectively) than in cases of HL (10 % and 5 % respectively). Furthermore, CNS impairment (mostly impairment of consciousness) at the time of the event was described in acidotic patients (11 %) but not in patients with HL.

Table 6.16 Symptoms reported at the time of the event by diagnosis

	LA (N= 46)		HL (N= 61)	
	N	%	N	%
Nausea	26	56.5	25	40.98
Vomiting	24	52.2	22	36.1
Abdominal pain	19	41.3	24	39.3
Weakness	16	34.8	11	18.0
Anorexia	14	30.4	17	27.9
Fatigue	13	28.3	18	29.5
Weight loss	13	27.7	17	27.9
Dyspnea	11	23.9	6	9.8
Tachypnea	6	13.0	3	4.9
CNS impairment	5	10.9	0	0
Jaundice	2	4.4	1	1.6
Other	18	39.1	16	26.2
Asymptomatic	0	0.0	10	16.4

Table 6.17 shows the univariate conditional logistic regression analysis performed for LA and HL as separate outcomes. Of note, even when there are apparent differences in the magnitude of the effect of some of the variables described before on each of the outcomes, it is important to state that in all cases effects were in the same direction and in almost all instances confidence intervals overlap. In addition, the study was not powered to assess differences between the two components of the outcome and therefore the

precision of the estimation of the impact of each analysed factor on HL and LA is quite poor.

Female patients were at much higher risk of developing LA (OR= 8.88; 95% CI= 2.57 – 30.64) than male participants. Although a trend in the same direction was noticed for HL, the effect of female gender did not reach statistical significance (OR= 1.94; 95% CI= 0.91 – 4.15). Similarly other factors show stronger impact in one of the outcomes, but as mentioned earlier no differences in the direction of any effect were found.

Table 6.17 Univariate analysis: Risk factors for LA and HL

Variable	Lactic Acidosis			Severe Hyperlactataemia		
	OR	P	95 % CI	OR	P	95 % CI
Demographics						
Age	1.004	0.805	0.97 - 1.04	1.06	0.002	1.02 - 1.09
Gender (female)	8.88	0.001	2.57 - 30.64	1.94	0.087	0.91 - 4.15
Mode of infection						
Heterosexual	5.96	0.003	1.80 - 19.75	2.39	0.044	1.02 - 5.56
IVDU	3.21	0.123	0.73 - 14.09	2.38	0.113	0.81 - 6.98
Blood Transfusion	6.16	0.164	0.48 - 79.70	2.84	0.215	0.54 - 14.85
HIV history						
Previous AIDS diagnosis	3.42	0.001	1.65 - 7.07	1.28	0.486	0.64 - 2.53
Previous adverse effects (ART)	2.16	0.053	0.99 - 4.72	4.40	0.002	1.76 - 10.96
Previous MT adv effects (ART)	1.9	0.073	0.94 - 3.83	2.00	0.049	1.00 - 3.98
Concurrent medical events						
Opportunistic infections	1.57	0.38	0.57 - 4.33	3.87	0.013	1.33 - 11.29
ART-associated adverse effect	4.50	< 0.001	2.08 - 9.74	2.91	0.003	1.44 - 5.88
ART-associated MT adv effect	5.39	< 0.001	2.52 - 11.54	6.95	0.001	2.30 - 21.07
CD4 (< 200)	7.06	< 0.001	2.64 - 18.87	1.89	0.139	0.81 - 4.40
ART exposure						
Current d4T exp	2.32	0.017	1.16 - 4.62	6.29	< 0.001	2.75 - 14.41
Current ddl exp	5.52	0.001	2.02 - 15.08	5.56	< 0.001	2.63 - 11.74
Current 3TC exp	0.57	0.119	0.28 - 1.16	0.11	< 0.001	0.04 - 0.28
Current AZT exp	0.23	0.009	0.08 - 0.70	0.26	0.004	0.11 - 0.65
Current TDF exp	10.28	0.031	1.24 - 85.21	1.70	0.398	0.49 - 5.87
Current d4T/ddl exp	3.25	0.020	1.20 - 8.79	25.12	< 0.001	5.97 - 105.67
Current AZT/3TC exp	0.18	0.007	0.05 - 0.63	0.30	0.010	0.12 - 0.75
Current ddl/TDF exp	6.61	0.096	0.72 - 60.86	4.00	0.100	0.77 - 20.87

6.2.4.1 Additional analysis assessing differences between risk factors for HL and LA

The presence of interactions between factors identified as significantly associated with the combined outcome and its two components (i.e. HL and LA) was also assessed. Interaction terms between components of the combined study outcome and each factor included in the final multivariate model for the combined model were defined and tested.

Three out of the five variables included in the model for the combined outcome showed significant interactions with LA. Female gender (OR= 4.58; P= 0.040; 95% CI= 1.07 – 19.57) and current CD4 count lower than 200 cell/ μ l (OR= 3.74; P= 0.046; 95% CI= 1.02 – 13.64) were shown as positively associated with LA in the univariate analysis. On the contrary, age at the time of the event was the only factor inversely associated with LA (OR= 0.95; P= 0.038; 95% CI= 0.91 – 1.00) although the magnitude of the effect was quite weak.

After adjusting for the other variables included in the model, the interaction term between female gender and LA was the only one which remained significant (OR= 11.51; P= 0.014; 95% CI= 1.65 – 80.32). Interestingly, the inclusion of such an interaction term did not influence the effect of any of the variables included in the model other than gender. All the factors included in the model remained almost at the same level of impact on the combined study outcome with the exception of female gender which lost its significance (OR= 1.78; P= 0.286; 95% CI= 0.62 – 5.12) when adjusted by the interaction term previously mentioned.

The analysis of the interactions suggests that female gender is strongly associated with LA but not necessarily with HL. Interestingly, female patients may not only be at higher risk of developing LA but may also be more likely to

develop severe episodes of HL. The median blood lactate at the time of the event was 7.0 mmol/l for the 17 female cases of HL (range= 5.4 – 13.2) whereas the same figure for the 44 male cases of HL was 5.8 mmol/l (range= 5.0 – 11.5) ($P= 0.075$). In addition, 52 % of female cases of HL had blood lactate levels equal or higher than 7 mmol/l at the time of the event but only 20 % of male cases had so ($P= 0.011$). On the other hand, all female cases of HL were symptomatic whereas 23 % of the male cases of HL were asymptomatic at the time of the event.

6.3 Outcome of the event

In this study the case fatality rate was 17.3 %, as 19 cases died as consequence of the episode. Sixteen of those who died were LA cases, a case fatality rate among acidotic patients of 32.7 % (16 deaths among 49 cases) whereas the case fatality rate among hyperlactataemia cases was 4.9% (3 deaths among 61 cases)..

Relevant information regarding re-exposure to ART drugs, further ADR and OI was collected from non-fatal cases as recorded in patients' notes. After the event, 33 non-fatal cases of LA were followed-up for a median of 36 months (IQR= 19 - 54) whereas the 58 non-fatal cases of HL were followed-up for a median of 33 months (IQR= 17 - 48).

6.3.1 Identification of risk factors for case fatality

Of the 19 fatal cases, eight (42.1 %) were women. The median age at the time of the event was similar for fatal and non-fatal cases 41.6 years (IQR= 38.1 – 49.01) and 42.8 years (IQR= 35.9 – 53.6) respectively.

Fatal cases were more likely to have had previous AIDS defining conditions. Sixteen out of the 19 fatal cases (84.2 %) had had previous AIDS diagnoses compared with 43 of the 91 non-fatal cases (47.3 %) ($P= 0.003$). However,

there was no significant difference in the nadir CD4 lymphocyte count between fatal and non-fatal cases. Thirteen fatal cases (81.3 %) and 56 survivors (74.7 %) had nadir CD4 lower than 200 cell/ μ l ($P= 0.577$). Fatal cases were more likely to have a concurrent OI at the time of the event (36.8 %) than non-fatal cases (13.2 %) ($P= 0.013$) but the proportion of those patients who died with a CD4 lymphocyte count lower than 200 cell/ μ l (55.6 %) at presentation was not significantly different from that in non-fatal cases (42.7 %) ($P= 0.320$).

Although there were no significant differences between fatal and non-fatal cases in the frequency of previous ART-induced ADR likely to be due to mitochondrial dysfunction (36.8 % and 38.5 % respectively; $P= 0.895$), fatal cases were more likely to have had previous abnormal blood lactate results reported in their clinical notes (47.4 %) compared to non-fatal cases (16.5 %) ($P= 0.003$). In addition, the median blood lactate level in those cases who died as a consequence of the episode of HL/LA at the time of their inclusion in the study was 8.33 mmol/l (IQR= 7.24 – 13.1), whereas for non-fatal cases it was 6.4 mmol/l (IQR= 5.4 – 7.8) ($P< 0.001$). Furthermore, 11 non-fatal cases had blood lactate levels lower than 5 mmol/l at the time of the event whereas all fatal cases had blood lactate levels higher than that figure. In fact 7 of the 19 of the fatal cases (37%) had blood lactate levels above 10 mmol/l.

Other laboratory abnormalities were also more frequently seen in those patients who died as a consequence of the event. All fatal cases had blood bicarbonate levels below 20 mmol/l whereas 30 of 79 (38 %) non-fatal cases had a normal blood bicarbonate at the time of the event ($P= 0.001$). Additionally, fatal cases were also more likely to have abnormal blood glucose 11 of 14 (78.6 %) than non-fatal cases 30 of 72 (41.7 %) ($P= 0.011$). Similarly, those patients who died were more likely to have abnormal alkaline phosphatase (15/19; 79 %) than survivors (37/80; 46.3 %) ($P=0.010$) and abnormal albumin (14/18; 77.8 % vs. 10/63; 15.9 % respectively; $P< 0.001$). However, there were no significant differences between fatal and non fatal cases in transaminases levels.

There were also no significant differences between fatal and non-fatal cases regarding the proportion exposed to different NRTIs other than d4T and 3TC. Sixty six non-fatal cases (72.5 %) were taking d4T at the time of the event whereas seven of the 19 fatal cases (36.8 %) were doing so ($P= 0.003$). Similarly, differences were found in the proportion of patients who were taking 3TC at the time of the event as 12/19 fatal cases (63.2 %) were taking 3TC whereas 28/91 non-fatal cases (30.8 %) were doing so ($P= 0.008$). In addition, differences were seen on ever exposure to either TDF or HU between fatal and non-fatal cases. About 6 of 19 (32 %) of the fatal cases had been exposed to TDF or HU at any point in the course of their HIV disease whereas only 12 % of non-fatal cases had been exposed to any of these drugs ($P= 0.033$). Two of these cases were exposed to both HU and TDF at some point in the course of their HIV disease.

In the univariate logistic regression analysis (Tab. 6.18), clinical LA was strongly associated with a fatal outcome ($OR= 9.37$; $95\%CI= 2.54 - 34.57$) and not surprisingly, high blood lactate (> 7 mmol/l, the median lactate level for cases in this study) ($OR= 8.94$; $95\%CI= 2.43 - 32.95$) and blood bicarbonate ($OR= 0.83$; $95\%CI= 0.75 - 0.92$) are strongly associated with case fatality (Table 6.17). Previous AIDS diagnosis ($OR= 5.95$; $95\%CI= 1.62 - 21.84$) and concurrent OI ($OR= 3.84$; $95\%CI= 1.26 - 11.68$) were also both associated with case fatality in the univariate analysis.

Interestingly, the only NRTIs exposure negatively associated with case fatality was d4T. In the univariate analysis, those patients who were receiving d4T at the time of the event were less likely to die ($OR= 0.26$; $95\%CI= 0.09 - 0.76$) compared to those who were taking ART combinations not d4T based. On the contrary, patients exposed to 3TC at the time of the event were almost four times more likely to die.

Table 6.18 Univariate analysis : Risk factors case fatality

Variable	Fatal (N=19)	Non-fatal (N=91)	OR	P	95 % CI
	N (%)	N (%)			
Lactic acidosis	16 (84.2)	33 (36.3)	9.37	0.001	2.54 - 34.57
Previous AIDS	16 (84.2)	43 (47.3)	5.95	0.007	1.62 - 21.85
Previous abnormal lactate	9 (47.4)	15 (16.5)	4.56	0.005	1.58 - 13.13
Current lactate > 7 mmol/l	16 (84.2)	34 (37.4)	8.94	0.001	2.43 - 32.95
Concurrent OI	7 (36.8)	12 (13.2)	3.84	0.018	1.26 - 11.68
Abnormal ALP	15 (79.0)	37 (46.3)	4.36	0.015	1.33 - 14.29
Abnormal albumin	14 (77.8)	10 (15.9)	18.55	< 0.001	5.05 - 68.10
Abnormal glucose	11 (78.6)	30 (41.7)	5.13	0.018	1.32 - 20.0
Current d4T exposure	7 (36.8)	66 (72.5)	0.22	0.004	0.08 - 0.62
Current 3TC exposure	12 (63.2)	28 (30.8)	3.86	0.010	1.37 - 10.84
Ever TDF exposure	6 (31.6)	11 (12.1)	3.36	0.040	1.06 - 10.65
Ever HU exposure	6 (31.6)	11 (12.1)	3.36	0.040	1.06 - 10.65

Finally, having a previous abnormal blood lactate result was also found to be associated with case fatality. However, blood lactate is not routinely measured in all participant centres and therefore, if such a test was performed in a particular patient it may suggest the presence of specific medical indication for that (e.g. symptoms). Consequently, it was considered inappropriate to include previous abnormal blood lactate as a variable in the multivariate model. Nevertheless, as described before, previous mitochondrialopathies were not more frequently seen among fatal cases.

After adjusting for other variables, the multivariate model developed for the study shows that those patients more severely ill were at higher risk of dying, which is not surprising. Patients with acidosis (OR= 10.1, 95%CI= 2.24 – 45.52), with blood lactate > 7 mmol/l (OR= 8.35, 95%CI= 1.93 – 36.14) and those with concurrent OI (OR= 7.64, 95%CI= 1.68 – 34.69) were at much higher risk of dying (Table 6.19).

Table 6.19 Multivariate model : Risk factors for case fatality

	Univariate			Multivariate		
	OR	P	95 % CI	OR	P	95 % CI
Lactic acidosis	9.37	0.001	2.54 - 34.57	10.1	0.003	2.24 - 45.52
Lactate > 7 mmol/l	8.94	0.001	2.43 - 32.95	8.35	0.005	1.93 - 36.14
Concurrent OI	3.84	0.018	1.26 - 11.68	7.64	0.008	1.68 - 34.69

It may be relevant to highlight that all asymptomatic cases (N= 10) included in this study had at least one of the factors associated with fatality in the multivariate model. Two of the asymptomatic cases had blood lactate > 7 mmol/l at the time of diagnosis. Similarly, five out ten asymptomatic cases had had previous abnormal blood lactate results and one of these asymptomatic cases had concurrent OI at the time of the event.

6.3.2 Further follow-up of non-fatal cases

Among the 91 non-fatal cases, 12 (13.2 %) developed further episodes of HL. and one further case developed a second episode of LA. The latter was a male patient who had previous episodes of pancreatitis and bone marrow suppression and was receiving a salvage ART combination which included d4T/TDF/enfuvirtide (T-20)/delavirdine at the time of his first episode of LA. The patient was admitted to hospital but his ART drugs were not stopped. He continued on medication until a month later when he developed an episode of pancreatitis and all drugs were stopped. Interestingly, the patient was restarted on anti-retroviral combination therapy including d4T two months after the pancreatitis. He received d4T/T-20/TDF/delavirdine for about seven months without any ADR reported in his clinical notes.

Three out of the 12 patients who developed further episodes of HL had previous episodes of LA. Two were female (16.7 %) and all 12 of them were restarted on ART using combinations which included NRTIs.

Four out of the 13 patients who developed further episodes of HL/LA (33.3 %) were exposed to d4T as part of their re-challenging ART combination, whereas 11 patients (14.1 %) of the 78 individuals who did not develop any further episode of HL/LA were also exposed to d4T ($P= 0.096$). Four out the 13 patients who had further episodes were re-exposed to ddl (33.3 %) while 14 (18 %) of the patients who did not have further episodes were also re-exposed to ddl including combinations ($P= 0.215$). Similarly, 10 of the patients who had further episodes were re-challenged with 3TC containing combinations (83.3 %) compared to 43 (55.1 %) of those who did not have further episodes ($P= 0.065$). Finally, 6 patients (50 %) of those who had further events were re-treated with AZT based combinations compared to 28 (35.9 %) patients among those who never had a further episode during the study follow-up ($P= 0.479$).

Of note, six out of the ten asymptomatic cases included in the study developed further episodes of HL/LA. Four of these patients were re-challenged with ART combinations including d4T, two of them including also ddl. The other two patients were treated with ddl but not d4T after their first event. Finally, two of the patients re-challenged with ddl were also treated with TDF, one of them in a combination with d4T.

Fifty four out of the 91 patients (59.3 %) who survived their episode of HL/LA developed further episodes of ART-associated ADR. Of these 20 individuals (22 %) developed further mitochondrial toxicities, including peripheral neuropathy ($N= 16$), pancreatitis ($N= 3$), myopathy ($N= 1$) and bone marrow suppression ($N=1$).

In a univariate analysis using logistic regression methods, the only factor found to be associated with further episodes of HL/LA was inclusion of d4T as part of the re-challenging ART combination ($OR= 3.81$; $P= 0.042$; 95% $CI= 1.05 - 13.78$). However, this calculation included the patient who developed a further episode of LA. As mentioned at the beginning of this section, that patient was not re-challenged with a d4T containing combination as he had

not stopped ART at the time of the initial event. If this patient is excluded from the analysis, then the previously noted association loses its significance ($P=0.108$) although the extent of the effect remains at the same order of magnitude ($OR=3.05$; 95% $CI=0.78-11.85$).

CHAPTER 7

Discussion for the case-control study on lactic acidosis and severe hyperlactataemia

As described in chapter five, the study has been designed to identify risk factors for HL/LA and therefore, that is the main subject of this discussion. However, a few elements regarding clinical and epidemiological characteristics of the included cases are also discussed here as well as issues concerning the outcome of the event, including factors associated with fatality.

7.1 Factors associated with HL/LA

As has been mentioned before, 110 cases of HL/LA were included in this study. Although the number of cases included in the study did not reach the target of 135 cases generated from the sample size calculation described in Chapter 5, this represents to our knowledge the largest case series and case control study of HL or LA carried out to date.

Because of the relative low incidence of LA and the strict case definition for HL used in this study, it was difficult to identify eligible patients for the study. In fact it took over a year to recruit the study population included in the study and it was necessary to include patients diagnosed in 19 centres from three different continents. Other factors however have also limited our capacity of recruiting a higher number of cases. Firstly, estimates of cases in some centres were too optimistic (i.e. over 10 initially proposed eligible cases were excluded from the study as they did not meet the study case definitions) and secondly, two overseas and three UK centres did not participate for various reasons. However, having included more than 80 % of the expected study population, it was possible to identify differences between cases and controls with a reasonable level of precision.

Furthermore, the calculation of the sample size required for this study was not based on conclusive data previously generated but on assumptions engendered from case-series, small studies (mostly retrospective), and the systematic review of published cases of LA (see Chapter 4) we performed earlier. Therefore, the result of the calculation, as in many other studies, was assumed to be more of a frame within which the research should be performed rather than an absolute goal which must be reached.

In addition to the issues previously mentioned, an objective of the study was to keep similar proportions of HL and LA cases in the study population to allow us to compare the two conditions. However, we made the assumption that it was likely that HL, as we defined it, was a precursor of LA. In fact, 49 of the 110 included cases (45 %) met the criteria for LA. As HL has been described as a more frequent event than LA (John *et al.* 2001) the intention to keep equivalent numbers of both outcomes in our study population made it even more difficult to reach the calculated sample size.

Finally, because of the difficulties and complications mentioned before in the process of identifying eligible cases and because of the logistic issues derived from the data collection it may have been probably unnecessary to extend the study until the calculated sample size was achieved, particularly in the light of the results already presented. Although many additional questions may be generated from the presented results, it is unlikely that all of these can be answered from retrospective data.

7.1.1 Clinical and epidemiological characteristics of cases

It has been suggested that female patients are more likely to develop at least some ART-related ADR. An observational study published few years ago shows that gender was independently associated with ART discontinuation because of toxicity. In that particular study, men were almost 50 % less likely than women to discontinue ART because of ADR (d'Arminio *et al.* 2000). Although the majority of cases included in our study were male, the proportion

of female patients among cases (36 %) was significantly higher than the proportion of female controls included in the study (18 %) ($P < 0.001$). Similarly, slightly more than 50 % of the cases included in the systematic review of published cases of LA (see Chapter 4) we performed were women. Likewise, Moyle *et al.* also found that female gender was over-represented among the nine cases with either severe HL (defined as blood lactate > 5 mmol/l) or LA they identified among 108 patients with raised blood lactate levels (Moyle *et al.* 2002). In Moyle's study, three out of the four cases of cases of LA (defined as blood pH < 7.35) and one of the five patients with severe HL were women. Furthermore, John *et al.* described five cases of severe HL (blood lactate > 5 mmol/l) in 516 patient-years of follow-up, two of whom were women (John *et al.* 2001). However, two of the male patients with severe HL described by John *et al.* were not receiving any ART drug at the time of the event, whereas the authors described the two female cases as the only "typical of NRTI-induced lactic acidosis/hepatic steatosis" they managed to identify. The last male patient identified by John *et al.* had a long history of alcohol abuse and was diagnosed with severe HL after acute alcohol intoxication.

Earlier studies published on the topic have not been able to identify any difference in the frequency of HL or LA events between male and female patients. For instance, a review on LA published by Mégarbane *et al.* including 40 cases published between 1991 and 1999 shows a male: female ratio of 1.18. (Megarbane *et al.* 1999). Nevertheless, cases included in the previously mentioned review were mainly reported from Western European countries or North America, where the prevalence of HIV infection is much higher in male individuals than in female. Therefore, similarities in the absolute number of cases reported in male and female patients may represent a relatively higher proportion of affected women. Finally, smaller studies have also failed to demonstrate any difference in the frequency of the event between men and women. A study on sustained HL (defined as two consecutive lactate readings > 2.1 mmol/l) which included only 11 cases, found that about 20 % of both cases and controls were female (Hocqueloux *et al.* 2003).

Because of three fatal cases of LA which were reported in pregnant women, the manufacturer of d4T and ddI produced a "dear healthcare provider" letter warning that pregnant women and their infants may be at higher risk of developing severe LA when exposed to the d4T/ddI combination (Carr 2003). The systematic review on LA we performed (see Chapter 4) included also three pregnant women who were more than 30 weeks of gestation at the time of the event (Arenas-Pinto *et al.* 2003). In our case control study we found only one pregnant woman among the cases and two of them among controls and therefore, this study failed to demonstrate any association between pregnancy and a higher risk for HL/LA.

It has also been suggested that older patients may be at higher risk of developing HL/LA. In our study the median age of cases was significantly higher (42.4 years; IQR= 36.1 – 52.5) than the figure calculated for controls (40 years; IQR= 35 – 47.1) ($P= 0.011$). However, conflicting results regarding age and its possible association with HL/LA have been reported. Cases of severe HL and even LA have been described in neonates exposed to prophylactic ART to prevent vertical HIV transmission (Blanche *et al.* 1999; Scalfaro *et al.* 1998). In addition the range of age among cases included in Mégarbane's review on LA was quite wide (0 – 68 years). Not surprisingly, age was not found to be associated with sustained but moderate HL in a small study which included only 11 cases (Hocqueloux *et al.* 2003). Nevertheless, in a study on peripheral lipoatrophy associated with lactic acidemia and liver dysfunction, the authors found that regardless of any other consideration and/or association, patients with lactic acidemia (defined as blood lactate > 2.0 mmol/l) were older than people with normal lactate ($P= 0.04$) (Carr *et al.* 2000). Furthermore, the Swiss Cohort Study has recently reported that older patients may be at higher risk of developing HL (defined as blood lactate > 2.4 mmol/l) but not necessarily severe HL (blood lactate > 5 mmol/l) (Imhof *et al.* 2005).

Although cases included in our study were diagnosed and managed in 19 different centres located in 13 different countries, the majority of the study population was white, a category which includes white Europeans, Americans and Australians (66 %). However, 42 % of included cases were described as non-white (i.e. black African, American or Caribbean, Asian or Hispanic) whereas the proportion of included non-white controls was 31 % ($P = 0.014$). As explained in Chapter 5, cases and controls were matched by centre, meaning that both were selected from the same population attending the centre and therefore were likely to share many characteristics, including ethnic background. In fact, a noteworthy ethnic homogeneity was noted in the population regularly seen at most participant centres. Therefore, the likelihood of randomly selecting a control with the same ethnic background as his or her respective case was quite high and consequently, matching by centre may imply overmatching by ethnicity as well. Taking these together, the impact of ethnicity on LA/HL may be underestimated in this study.

Furthermore, most of the cohort or case-control studies on HL, LA or both published to date did not contain information regarding ethnicity of included participants but all of them have been performed in centres where population homogeneity is also a prominent characteristic (Imhof *et al.* 2005; John *et al.* 2001; Moyle *et al.* 2002). Nevertheless, a recently presented prospective study shows that black Africans may be more likely to develop severe hyperlactataemia (defined as blood lactate > 5 mmol/l). In that French study, 574 patients who started their first line ART combination over the study period were followed. Interestingly, about 20 % of the study population was from Sub-Saharan countries and more than half of these were black female patients. Six out of the 67 black African female participants developed HL over the study period and three had severe HL. The authors conclude that black women may be at much higher risk of developing severe HL compared to men (OR= 18.2; 95% CI= 3.7 – 88.7). Of interest, two of the three cases of severe HL described by Gerard had CD4 counts lower than 50 cell/ μ l at base-line (Gerard *et al.* 2005).

Unfortunately, there are no conclusive data on the incidence of HL/LA in settings other than predominantly Caucasian populations. There are a few large clinical trials currently on going in African countries. In addition, several developing countries in Latin America and the Caribbean as well as a few South-East Asian countries have national AIDS control programs which include ART delivery. Some of these programs have been in place for several years now. Clinical trials and observational cohort studies could be used to assess the incidence of HL/LA, as well as other ART-associated ADR, in predominantly non-white populations.

It has also been repeatedly suggested that patients with more advanced HIV-disease may be at higher risk of developing ART-associated ADR such as lipoatrophy (Joly *et al.* 2002). In this study, 54 % of the included cases had been previously diagnosed with at least one AIDS defining illnesses. Furthermore, cases were certainly more likely to have more advanced HIV disease than controls. Median nadir CD4 lymphocyte count for cases was significantly lower than the figure for controls ($P < 0.001$). As was, the median CD4 lymphocytes count at the time of being included in the study ($P < 0.001$).

A small case-control study on LA (defined as blood lactate > 5 mmol/l and arterial pH < 7.38) also found that cases were more likely to have nadir CD4 lymphocyte count lower than 250 cell/ μ l than controls ($P = 0.03$). However, the authors did not find any significant difference in the CD4 lymphocyte count between cases and controls at the time of the event ($P = 0.10$) (Bonnet *et al.* 2003). Also, a recently published report from the Swiss Cohort Study also showed that higher CD4 lymphocyte count may be a protective factor for HL (defined as blood lactate > 2.4 mmol/l). However, there was no significant difference between cases and controls regarding the level of CD4 lymphocyte in peripheral blood at the time of the event (Imhof *et al.* 2005). In the same way, other prospective studies have failed to demonstrate any association between CD4 lymphocyte count and the likelihood of developing HL (Hocqueloux *et al.* 2003).

Differences in the case definition and particularly the number of cases in the studies mentioned in the previous paragraph may explain, at least in part, the contradictory results with our findings. The study published by Bonnet et al. included only nine cases with a median CD4 lymphocyte count of about 200 cell/ μ l and a very wide range (31 – 707 cell/ μ l). In addition, the study published by Hocqueloux et al. also included only 11 cases. The case definition they used required the confirmation of sustained abnormal blood lactate but the cut-off used (2.1 mmol/l) allowed the inclusion of patients with very mild episodes of HL. In fact, the baseline blood lactate level of the 11 cases ranged from 2.2 to 5.8 mmol/l and none of them had LA. Therefore, these patients were very different to the group of cases included in our study. Both the studies published by Bonnet and Hocqueloux included a very small number of cases and therefore, the absence of any association between this and any other exposure or potential risk factor and the study outcome may reveal just the lack of power of such studies to do so, rather than the lack of real associations.

The Swiss Cohort report includes a large population (N= 1,566 participants) but only 49 cases of severe HL were identified (blood lactate > 5 mmol/l) in there. However differences from our study were that their case definition allowed patients with only one blood lactate measurement above the cut-off to be included as cases and only four patients developed LA.

Although few case-series, cross-sectional, case-control and cohort studies on HL or LA have been published since the late nineties to date, there is still not a universally accepted case definition for hyperlactataemic conditions in HIV-infected patients on ART. John et al. have proposed to classify lactate metabolism abnormalities in this group of patients by dividing them into three categories: compensated asymptomatic hyperlactataemia, symptomatic hyperlactataemia or hepatic steatosis without lactic acidosis and decompensated lactic acidosis or hepatic steatosis (John & Mallal 2002). In their review, John et al. suggest that severe hyperlactataemia may have different implications when patients are acidotic. However, a more recent

review suggests the unification of all cases of abnormal blood lactate conditions in HIV-infected patients into only one clinical entity “lactic acidemia” referred to in chapter 4 (Carr 2003). This approach considers that risk factors and therefore predisposing conditions may be the same for developing any form of lactate metabolism impairment associated with NRTIs exposure. That approach has been supported as well by an International AIDS Society-USA Panel (Schambelan *et al.* 2002).

In any case, both approaches include symptoms associated with elevated blood lactate as part of the criteria for grading or classifying the complication. However, all case reports, case-series, prospective or retrospective studies published to date suggest that symptoms associated with HL/LA are quite non-specific and frequently seen in HIV-infected patients not affected by these conditions. In our study, as in all other previously published studies, most patients had gastrointestinal (65 %) or constitutional symptoms (57 %) whereas severe symptoms such as CNS impairment were described in about 5 % of the included cases. However, all five cases with CNS abnormalities at the time of the event were patients with LA.

Of note, 10 out of the 61 included cases of HL (16 %) were apparently asymptomatic at the time of being diagnosed with the event. By definition, all cases of HL included in our study had confirmed blood lactate levels above 5 mmol/l. Interestingly, John *et al.* have pointed out that patients with asymptomatic hyperlactataemia usually have mild to moderate blood lactate elevations (2 – 5 mmol/l) with only occasional or intermittent elevations of blood lactate over 5 mmol/l (John & Mallal 2002). This observation was based on results of a longitudinal study performed by the same group where all five patients with blood lactate > 5 mmol/l were highly symptomatic (John *et al.* 2001). Furthermore, the management guidelines for lactic acidemia proposed by Carr and by the International AIDS Society-USA Panel suggest implementing medical interventions in those patients with confirmed blood lactate between 5 – 10 mmol/l only when symptoms likely to be related with the condition are present (Carr 2003; Schambelan *et al.* 2002).

Nevertheless, because of the retrospective nature of this study, it is not possible to exclude the possibility of mild symptoms not being recorded. Mild symptoms may have been ignored by either patients or clinicians and therefore not reported in patients' medical notes. However, it is very unlikely that moderate to severe symptoms have been ignored. Similarly, symptoms likely to be associated with LA such as CNS impairment or changes in the respiratory rate are difficult to be uncared for and therefore even when we can not be absolutely sure about the absence of symptoms in this small group of patients, we can assume that if something they may have had noting but mild, unspecific physical complains.

In addition, the study published by Hocqueloux *et al.* also found a high proportion of asymptomatic patients (7/11) among their cases of HL. Noteworthy, the latest study included mainly cases of mild-moderate HL (as HL was defined as blood lactate > 2.1 mmol/l). In fact, only one patient had blood lactate > 5 mmol/l at the time of being included in that study. Of note, all four symptomatic cases described by the authors had blood lactate levels lower than 5 mmol/l. Three of these four individuals had symptoms not likely to be related to HL. Nevertheless, the fourth patient was a woman who did not have any other obvious explanation for her symptoms (fatigue and vomiting) at the time of the event and her blood lactate at inclusion was 4.5 mmol/l (Hocqueloux *et al.* 2003).

Taking all together, one could state that even when the presence of severe symptoms such as CNS impairment or respiratory rate alteration are usually associated with cases of LA, the presence of constitutional symptoms with or without gastrointestinal impairment may not be present in all cases of severe and sustained HL. In addition, cases of symptomatic mild HL have also been described. Even more important, it can be noted from the results presented in this study that asymptomatic cases of severe and sustained HL may not be considered as less severe than symptomatic cases. Even when none of the asymptomatic cases died as consequence of the event in our study (see

section 7.3), six of them (60 %) had at least one of the factors associated with case fatality according to the multivariate analysis shown in section 6.3.1. Furthermore, six of the ten asymptomatic cases developed further episodes of HL once re-challenged with (or kept on) NRTIs.

The overall median blood lactate level at the time of diagnosis of the episode of HL/LA in our study was about 7 mmol/l (IQR= 5.5 – 8.1), but cases with blood lactate levels up to 20 mmol/l were included. Nevertheless, such a figure is somewhat lower than the median blood lactate observed among cases included in our systematic review discussed in Chapter 4 (median= 10.5 mmol/l; IQR= 7.0 – 17.3). However, a significant proportion of the cases included in our review (50 %) were diagnosed during the nineties, when the level of awareness of clinicians for this condition was relatively low and consequently the diagnosis of LA was likely to be delayed. By contrast, only 17 (16 %) of the cases included in this study were diagnosed before the year 2000 and some of them were diagnosed as part of routine blood lactate measurements, leading to early diagnosis and prompt interventions. Higher blood lactate concentrations have been described in cases of LA severe enough to be admitted to intensive care units (median= 20.1 mmol/l; range= 14 – 28) (Bonnet *et al.* 2003). There were only 9 cases in the last study, seven of whom died, so their sample was biased towards the extreme severity range of the condition.

Abnormal laboratory results reflecting several organs involvement have been reported consistently in patients with both severe HL and LA. For instance, abnormal liver function tests (LFT) have been reported quite often in cases of HL/LA. In fact, it has been proposed that the targeted organ for severe HL/LA associated with NRTIs exposure is the liver. Several case-series and case reports of LA have shown liver involvement. Hepatic steatosis has been associated with LA (Freiman *et al.* 1993; Miller *et al.* 2000) and with symptomatic HL (Gerard *et al.* 2000). Although, fatal episodes of hepatic failure have been described in patients with NRTI-associated LA events (Lai *et al.* 1991; Carr *et al.* 2001) the commonest pattern of presentation includes just

mild to moderate elevations in transaminases (Carr 2003; Stenzel & Carpenter 2000). In this study, even when cases were more likely to have abnormal ALP and ALT results than controls ($P < 0.001$), the median times over the upper limit of normal among the 62 cases with abnormal ALT was 1.96 (IQR= 1.5 – 2.7) which was quite similar to the pattern of ALT elevation seen among the 43 controls with abnormal ALT at the time of being included in the study (median= 1.45; IQR= 1.2 – 2.04).

Furthermore, it has been suggested that patients affected by HL/LA are more likely to have concurrent chronic viral hepatitis (John & Mallal 2002). However, in our study there were no differences in the proportion of cases and controls with concurrent chronic hepatitis B or C (OR= 1.29, 95% CI= 0.44 – 3.76 and OR= 1.88, 95% CI= 0.88 – 4.02 respectively). Similarly, a small case-control study on sustained HL (defined as blood lactate > 3.5 mmol/l in at least two consecutive occasions) could not find any difference between the proportion of cases and controls with concurrent chronic hepatitis B either (Datta *et al.* 2003). Also, a study involving patients with LA also failed to demonstrate any higher frequency of chronic hepatitis B or C co-infection among cases when compared to controls (Bonnet *et al.* 2003).

Similarities between proposed risk factors of both the proposed “lactic acidosis/hepatic steatosis syndrome” and the most frequent cause of abnormal LFT in general adult population, which is non-alcoholic fatty liver disease (NAFLD) are quite remarkable (Miller *et al.* 2000; Day 2002). Moreover, hepatic steatosis has been reported in many cases of NRTI-associated LA (see Chapter 4). In our study we could not find any difference between cases and controls in the frequency of hepatic steatosis diagnosed by imaging studies. In fact, almost 50 % of the 34 cases in whom abdominal ultrasound scanning was performed at the time of the event had images suggestive of fatty infiltration. A very similar proportion of the 11 controls in whom abdominal ultrasound was performed had the same type of echo-pattern. The proportion of abnormal results among control patients is almost certainly affected by a selection bias as liver ultrasound was probably carried

out only in those patients showing evidence of hepatic impairment and therefore, the group of patients with imaging study results may not be representative of the general population. Furthermore, above 30 % of the abdominal ultrasound scans performed in patients with HL/LA at the time of the event were reported as normal. Therefore, it is not possible from these data to draw any firm conclusions regarding the frequency of fatty liver infiltration in patients who develop episodes of HL/LA.

Moreover, factors other than concomitant viral hepatitis or the effect of ART on hepatocytes can not be completely excluded. For instance, in this study cases were more frequently described in their clinic notes as having had previous serious alcohol consumption habits than controls (23 % vs. 11 % respectively; $P= 0.004$), and that may also play a role in the pathogenesis of abnormal LFT results seen in cases. However, it is not possible to draw conclusions on alcohol consumption and its possible contribution to abnormal LFT results in our study as alcohol consumption habits were not universally described in patients' notes and no details of this were recorded in the study proforma. In addition, this may be an example of reporting bias, which is one of the potential limitations of this study (see section 7.4): i.e. more information with respect to past and current medical history is reported in cases than in controls.

Cases included in this study were also more likely than controls to have abnormal blood glucose and amylase levels. Although there was a significant proportion of missing data on these variables, it was possible to notice that the differences between cases and controls were statistically significant ($P< 0.001$ and $P= 0.034$ respectively). Patients with HL/LA have been found to have higher blood glucose levels than controls in several previous studies (Carr *et al.* 2000; Moyle *et al.* 2002). Lo *et al.* have recently proposed that insulin resistance may be an additional mechanism leading to abnormal lactate values in patients exposed to NRTIs therapy. They found that after adjusting for several factors, including duration of NRTIs exposure, insulin resistance (measured by homeostasis model assessment -HOMA-IR-) was significantly

associated with blood lactate levels (Lo *et al.* 2005). In the same way, another prospective study has recently shown that insulin resistance as well as fasting hyperinsulinemia in HIV-infected men are related to cumulative NRTIs exposure (Brown *et al.* 2005). About 10 % of cases but less than 1 % of controls included in our study had previous diagnosis of diabetes mellitus ($P < 0.001$). A significantly higher proportion of cases (13 %) had previous episodes of NRTI-associated pancreatitis than controls (1 %) ($P < 0.001$). Therefore, it is also possible that, at least in a sub-set of the included cases, hyperglycaemia may precede the episode of HL/LA. However, as explained before, it is not possible to draw any robust conclusion from these data as reporting bias may be an issue.

In the study published by Moyle *et al.* on HL/LA the authors noted that all four cases of LA they described had concurrent bacterial infections (i.e. respiratory or urinary tract infections) at the time of the event (Moyle *et al.* 2002). Although it was not recorded in the study proforma, the possibility of concurrent bacterial infections in the cases included in this study can not be disregarded. About 17 % of the included cases had leukocytosis at the time of being included in the study, and almost 80 % of them had neutrophils count above the normal range. In addition, almost 12 % of the cases had neutropenia, three of them with neutrophil count $< 1 \text{ cell} \times 10^9/\text{l}$. In addition, about 20 % of the non-fatal cases of LA included in this study were treated with antibiotics as part of the event management interventions.

7.1.2 Mitochondrial function

Very limited information was recorded on mitochondrial function or morphology at the time of the event for this study. In fact only three patients out of the 110 included cases had mitochondrial function tests performed.

Two of the included patients had normal mtDNA content in muscle cells, even when one of them had mtDNA depletion in PBMC at the time of the event. It has been documented in both clinical and in vitro studies that AZT and

AZT/3TC or AZT/ddI combinations can lead to mitochondrial dysfunction without affecting mtDNA content (Szabados *et al.* 1999; Miller *et al.* 2003; Setzer *et al.* 2005). Of note, the patient with normal mtDNA content in muscle cells, but with no studies performed on PBMC was receiving AZT/ddI at the time of the LA event. However, the patient with normal mtDNA content in muscle cells but mtDNA depletion in PBMC was taking ddI/TDF at the time of the HL event.

Although it was not possible to collect a great deal of information on mitochondrial function in patients with HL/LA included in this study, the data presented on these three cases does illustrate the spectrum of mitochondrial dysfunction in this type of patients. Mitochondrial respiratory chain dysfunction may be evident in patients with HL/LA even when mtDNA depletion is not. Furthermore, different cell types may express different patterns of mitochondrial dysfunction in the same patient at the time of the event, which suggest a tissue specificity of NRTI-induced adverse effects (Cherry *et al.* 2002; Olano *et al.* 1995). Finally, patients with HL/LA can have impairment of all respiratory chain complexes, including complex II which is completely encoded by nuclear DNA. The two patients who had significant reduction in the activity of complex II in at least one cell type were assessed at the same centre. Another patient with symptomatic HL who was also evaluated at that centre has been reported as having significant reduction in the activity of all respiratory chain complexes as well as mtDNA depletion at the time of the event. Interestingly, that patient's mitochondrial parameters all came back to normal once therapy was stopped and the patient became asymptomatic (Lopez *et al.* 2002). Similarly, the included patient who had mitochondrial studies done only in muscle cells also had a repeated muscle biopsy 14 months after the event where not only functional tests but also mitochondrial morphology was restored to normal (Miller *et al.* 2003). In this patient at the time of the event only minor mtDNA deletions were found but no other alterations in mtDNA structural integrity. Nevertheless, major deletions (Walker & Venhoff 2001) and different cumulative type mutations, affecting

respiratory chain complexes components synthesis have been reported in vitro and in vivo (Martin *et al.* 2003).

7.1.3 Risk factors for lactic acidosis or severe hyperlactataemia

Substantial evidence has been presented over the last 15 years about the association between NRTIs exposure and mitochondrial dysfunction. In fact, even when several proposed NRTI-induced ADR have been attributed to mitochondrial toxicity (see Chapter 2), LA has been considered as the proof of the concept for the proposed association (Brinkman 2001).

HL/LA has been described in patients exposed to almost all NRTIs drugs currently in use, with the clear exception of ABC and perhaps 3TC. Although combination therapy has been the standard of care for more than a decade now, it is important to note that ART combinations are not randomly allocated, but follow both historical and biological rationale. Therefore, it is very difficult to assess the effect of any individual drug on lactate metabolism and therefore on mitochondrial function in vivo.

Furthermore, most of the studies performed to date on mitochondrial dysfunction associated with NRTIs exposure have focused on the analysis of drug exposures at the time of the event, without considering either the length of exposure or the implications of previous exposures to different ART drugs on the studied outcome. An exception was the study published by Carr *et al.* in which patients with LA received d4T for longer periods than controls (Carr *et al.* 2000). Some studies have also highlighted the possible role of specific NRTIs combinations on the likelihood of developing ADR such as HL/LA (Imhof *et al.* 2005; Boubaker *et al.* 2001).

In our study we assessed NRTIs exposure using three different approaches. Previous exposures to different NRTIs as well as exposures at the time of the event were both assessed. In addition, duration of the exposure to all ART drugs each patient had been on, i.e. those being taken at the time of the event

and those previously prescribed were both analysed. Finally, as it is difficult to separate individual NRTIs effects, the analysis performed using single drug exposure as variable was repeated using the most frequent NRTIs combinations seen in the study population.

We were unable to identify any association between previous exposure to any NRTIs drug and the study outcome. As has been discussed previously (see Chapters 4 and 5), evidence suggests that exposure to d4T is a strong inducer of mitochondrial dysfunction (Kakuda 2000). It has also been proposed that d4T exposure may be considered as a marker of long term exposure to NRTIs because a significant proportion of patients on d4T would have been previously exposed to AZT. In fact, 36 out of the 73 patients (49 %) who were on d4T at the time of the event in our study had been previously exposed to AZT. However, as stated before, previous exposure to AZT, even among those who were currently receiving d4T was not associated with the study outcome. Furthermore, no significant difference was found in the risk of developing HL/LA between patients receiving d4T who had or not had previous courses of treatment with AZT. Similarly, John et al. reported in a study which included 10 cases that the association between d4T exposure and HL/LA was not confounded by previous AZT exposure or by longer history of NRTIs use (John *et al.* 2001).

Current exposure to d4T and ddl were strongly associated with the development of HL/LA outcome. Of note, d4T was selected as the main exposure for our study design based on several previous studies, including our systematic review presented in Chapter 4, where d4T use was strongly associated with either LA or HL (Arenas-Pinto *et al.* 2003; Boubaker *et al.* 2001; John *et al.* 2001). Nevertheless, the association between ddl exposure with HL/LA has also been highlighted. Moyle et al. have reported that patients receiving ddl based ART combinations had twice the risk for HL (defined as blood lactate > 2.4 mmol/l) compared with those not exposed to that drug. They also found that treatment with ddl was overrepresented among patients with severe HL or LA (defined as blood lactate > 5 mmol/l) (Moyle *et al.* 2002).

Nevertheless, in a further matched case-control study performed by the same group matching by ddl exposure, in the same study population d4T exposure became independently associated with HL (defined as blood lactate > 3.5 mmol/l) (Datta *et al.* 2003).

Concomitant exposure to both d4T and ddl has been frequently seen in patients with HL/LA. In this study more than 40 % of the cases of HL/LA were receiving a combination ART including d4T/ddl at the time of the event whereas the proportion in controls was 12 %. Furthermore, in the multivariate model exposure to d4T/ddl combination was associated with a six fold increase in the risk of developing HL/LA when compared with those patients on ART combinations other than d4T/ddl.

The most recent report from Swiss Cohort Study on the subject highlights that the exposure to d4T is associated with the development of severe HL (defined as blood lactate > 5 mmol/l) only when it is administered in combination with ddl (HR= 6.65; 95% CI= 2.7 – 16.3). Furthermore, even when the statistical significance of the association between ddl without concomitant d4T exposure and severe HL was lost in the multivariate model (HR= 2.95; 95% CI= 0.8 – 10.4), the univariate analysis showed a significant increase in the risk of developing severe HL among those patients exposed to ddl but not to d4T (HR= 4.46; 95% CI= 1.3 – 15.3) (Imhof *et al.* 2005). In our study, the increase in risk attributable to ddl was significant even in the absence of concurrent d4T exposure (OR= 4.06; P= 0.017; 95% CI= 1.29 – 12.80). In the same way, patients who were on d4T with no concomitant exposure to ddl appeared also at significant higher risk of developing HL/LA in the univariate analysis (OR= 2.92; P= 0.015; 95% CI= 1.23 – 6.93) when compared to patients not on d4T containing regimes. It is important to mention that a significant proportion of the cases who were on d4T (64 %) were also taking ddl simultaneously whereas only 34 % of the controls who were on d4T (N= 27) were taking ddl at the same time. In addition, 64 % of the cases who were on ddl were taking that in combination with d4T (N=47).

Nevertheless, the actual proportion of patients exposed to ddl at the time of the inclusion in the first study on HL reported from the Swiss cohort was 22 % which was not very different from the overall proportion of patients currently exposed to ddl in a smaller prospective study on sustained HL (defined as blood lactate > 2.1 mmol/l; N= 11 cases) where authors found a significant association between ddl exposure and HL. In that study authors suggested that the association between ddl exposure and HL is to some extent higher than the association between d4T and the same outcome, even when no risk analysis was presented (Hocqueloux *et al.* 2003). In our study the overall proportion of current exposure to ddl was 36 %, and certainly the association between ddl exposure and HL/LA was consistently found irrespective of the approach used to analyse the effect of NRTIs on the likelihood of developing HL/LA.

In contrast, the study performed by John *et al.* did not find any association between ddl exposure and HL in the Western Australian Cohort (N= 349) (John *et al.* 2001). However, the actual number of patients exposed to ddl in that cohort was very low. Only 44 out of 349 participants (13 %) in the previously mentioned study were receiving ddl. However, the proportion of people on ddl in the study published by Moyle *et al.* was fairly similar to that figure as it was about 16 % and they did find an association between ddl exposure and HL (Moyle *et al.* 2002).

Although different studies have shown slightly different results on the association between d4T, ddl and the combination of these two and HL/LA, it has been constantly demonstrated that exposure to these drugs increases the risk of developing HL/LA. In addition, *in vitro* data also support these findings. Results presented in chapter 6 show that exposure to both d4T and ddl are independently associated with HL/LA. In addition, when exposure to the combination of these two drugs (d4T/ddl) is analysed, the magnitude of the effect on HL/LA looks even stronger than the effect of any drug considered individually, suggesting an additive effect. It looks like exposure to d4T, ddl or d4T/ddl is associated with a real rise in the risk of developing HL/LA.

However, to assess accurately the magnitude of the effect of each drug is extremely complicated as drugs are always prescribed in combination and therefore, there is not an easy way to control for the effect of other drugs included in frequently used combinations.

Conversely, in our study patients exposed to AZT/3TC containing combinations were at much lower risk of developing HL/LA. In fact, after adjusting by other factors included in the multivariate model, exposure to AZT/3TC shows a “protective” effect for HL/LA (OR=0.35; 95% CI= 0.14 – 0.89) when compared to those patients receiving different NRTIs backgrounds. AZT and d4T are not prescribed together because of the interactions described between these two. One would assume therefore that being on treatment with AZT containing combinations may protect against HL/LA simply because d4T can not be added to AZT containing regimes. Of interest, only one case and seven controls were receiving AZT/ddI containing combinations at the time of their inclusion in the study.

A few clinical and in vitro studies have suggested that the duration of the exposure to NRTIs, and particularly to d4T, may play a significant role in mitochondrial dysfunction (ter Hofstede *et al.* 2000; Walker *et al.* 2002). Harris *et al.* have found that a longer duration of d4T exposure was associated with a moderate (12 % per quarter year) increase in the likelihood of abnormal random venous lactate result (Harris *et al.* 2002). Similarly, a prospective study on mitochondrial genetics in HIV-infected patients found that duration of NRTIs exposure was the only factor associated with mtDNA depletion (McComsey *et al.* 2005a). Similarly, a few clinical studies have suggested that longer exposure to both d4T and ddI may be associated with HL/LA (Carr *et al.* 2000; Manfredi *et al.* 2003) in contrast to others who have not found any association between duration of the exposure to any NRTIs and the likelihood of developing HL (Bonnet *et al.* 2003).

Interestingly, cases included in our study were more likely to receive d4T for shorter time periods than controls. As mentioned in chapter 6, the odds of

developing HL/LA in those patients on d4T decline by about 3 % each month. Actually, patients exposed to d4T for periods longer than 15 months were at significant lower risk of developing HL/LA (OR= 0.27; 95% CI= 0.11 – 0.68) when compared to patients exposed to d4T for shorter periods of time. Furthermore, the median d4T duration for cases immediately before the event was about 6 month shorter than the figure calculated for controls (11.74 and 18.42 months respectively). This could be interpreted as a kind of “survivor bias” as those patients exposed to d4T who did not develop any serious ADR could keep taking ART combinations including d4T whereas those patients who developed a severe d4T-associated ADR (such as severe HL or LA) generally are switched away from such a drug. Therefore, the duration of the exposure to d4T among controls in this study may be longer than the duration of the exposures among cases because the study outcome censors further d4T exposure.

Nevertheless, previous studies have suggested that patients with borderline mitochondrial function or those with sub-clinical mitochondrial impairment may be at higher risk of developing NRTI-induced clinically evident mitochondrial dysfunction (Moyle 2000). Therefore, such individuals may be particularly susceptible to the effect of any mitochondrial toxic drug, including NRTIs and particularly d4T. For instance, a few reports have suggested an association between late onset of Leber’s hereditary optic neuropathy and NRTIs exposure in HIV-infected patients (Shaikh *et al.* 2001; Luzhansky *et al.* 2001).

A recently published study on risk factors for peripheral neuropathy (PN) in HIV-infected patients on ART has shown that those patients belonging to mitochondrial haplogroup T, a common haplogroup described in European populations, are at higher risk of developing PN when compared to people showing different haplogroups. The study, which was a sub-study of the ACTG trial 384, was limited to white patients. The effect of haplogroup T was stronger among those randomised to receive d4T/ddI (Hulgan *et al.* 2005). Similar observations have been made outside of the HIV field in a small study on ototoxicity attributed to cisplatin. Patients belonging to the mitochondrial

haplogroup J, a rare European haplogroup, were overrepresented among those with impaired hearing when compared to exposed patients with normal hearing (Peters *et al.* 2003).

Taken all together, one could hypothesise that a particular group of individuals may be at much higher risk of developing serious NRT-induced mitochondrial dysfunction. They may already have a particular genetic background which may or may not lead to sub-clinical mitochondrial dysfunction, but which facilitates a deleterious effect of NRTIs on mitochondrial function. If this is the rationale, relatively short exposure periods to any mitochondrial toxic drug may be enough to induce clinically evident mitochondrial dysfunction in those patients with pre-existing inherited conditions.

Furthermore, although the study was not powered to examine the effect of ethnicity on the study outcome, there was a suggestion that ethnicity may be a risk factor. As mentioned before, Gerard *et al.* have reported that black African women may be at much higher risk of developing severe HL when compared with white women (Gerard *et al.* 2005). Similarly, our data suggest that the occurrence of HL/LA may be more frequent in non-white populations.

Nevertheless, the data presented in our study and taking into account those from other studies, cannot allow one to reach any definitive conclusion regarding the mechanisms for the proposed susceptibility in a particular group of individuals. Studies specifically designed to answer that question are urgently needed. A better identification of those individuals at significantly higher risk of developing serious NRTI-induced mitochondrial dysfunction, such as HL/LA, may help clinicians to make more appropriate choices when starting a specific patient on ART. This may be particularly relevant in resource limited settings where ART options are restricted, laboratory support for monitoring therapy is scanty and confidence of people on HIV-control programs may be seriously affected by the occurrence of serious ADR (Katabira 2005). Proper identification of risk factors for serious ADR, such as HL/LA, and particularly elucidation of the proposed inherited predisposition

may result in a significant step not only in the prevention of morbidity and even mortality associated to HIV-infection management, but also in boosting community acceptance and support in HIV control programs in developing countries.

Advanced HIV disease has also been proposed as an independent risk factor for NRTI-induced ADR (Joly *et al.* 2002). In our study low nadir and CD4 count at the time of the event (< 200 cell/ μ l) were significantly associated with the study outcome in the univariate analysis (OR= 4.38, 95% CI= 2.08 – 9.22 and OR= 3.63, 95% CI= 1.94 – 6.83 respectively). Furthermore, CD4 count at the time of the event was independently associated with HL/LA in the multivariate analysis. Therefore, one could assume that our study may be comparing a group of patients with advanced HIV disease, more likely to develop ADR anyway with a group of people with better controlled HIV disease. The latter may have started ART earlier and therefore would have better control of viral replication and in consequence lower risk of developing ADR. In fact, 76 % of the 91 cases with information on nadir CD4 count had less than 200 cell/ μ l. Out of these 91 cases 85 individuals had also current CD4 counts reported in their notes. About 40 % of the included cases had both low nadir CD4 and low current CD4 count whereas only 18 % of controls with information regarding these values had both nadir and current CD4 counts lower than 200 cell/ μ l.

Certainly from the data collected for the study, it is not possible to find out if these cases with both low nadir and current CD4 counts just had a poor response to ART and remained severely immunosuppressed during their exposure to ART drugs or they just had a latter decline in their control of viral replication. However, in any case, there is a consistency in the finding of an association between advanced HIV disease markers and the likelihood of developing NRTI-associated ADR. In the context of the other results presented here, it appears that the use of specific drugs, such as d4T and ddI in developing countries where patients tend to start ART at advanced stages of the HIV disease should be considered as an important issue. Financial

and public health variables need to be taken into account but also so should safety issues.

Another relevant finding in our study is the strong association between female gender and the study outcome. Previous studies have suggested that HL/LA may be more frequently seen in female patients (Arenas-Pinto *et al.* 2003). Nevertheless, the precise mechanism for that predisposition is not completely understood. Female patients exposed to AZT/3TC show a significantly higher intracellular concentration (assessed in PBMC) of the respective NRTIs triphosphate metabolites, which may induce a higher degree of toxicity (Anderson *et al.* 2003). However, no data on d4T or ddI metabolites have been presented. In addition, other factors may also play a significant role. Hormonal variations, assessed through estradiol and progesterone infusion in a mice model, may lead to liver mitochondrial dysfunction which terminates in acute hepatic steatosis (Grimbert *et al.* 1995). Finally, other chronic conditions recently associated with mitochondrial dysfunction, such as Alzheimer's disease, have been found to be more prevalent in women than in men. This difference in prevalence between genders has also been suggested to be a consequence of greater longevity. However, several epidemiological studies have noticed that Alzheimer's disease is still more frequent among women than in men even when other factors are controlled for (Barclay *et al.* 1985; Rocca *et al.* 1990). Furthermore, an interesting in vitro study with teratocarcinoma cells has suggested that mtDNA-related mitochondrial dysfunction may play a significant role in cellular degeneration in Alzheimer's disease (Cardoso *et al.* 2004). Therefore, it is possible that women or a specific group of women are at higher risk of developing clinically evident mitochondrial dysfunction not only when exposed to mitochondrial toxic drugs, but also as consequence of other physiological and pathological processes.

It has also been suggested that women, particularly those from ethnic minorities living in developed countries, tend to get access to regular HIV medical care late in the course of their HIV disease compared to men (Anastos *et al.* 2005; Clark 2005; Currier *et al.* 2000). So, it is possible that the

effect of advanced HIV-induced immunosuppression may be stronger among female patients exposed to ART as they may have been started on ART at a more advanced stage of their HIV disease. However, in our study male and female cases show very similar pictures of HIV disease progression both at base-line (nadir CD4 < 200 cell/ μ l was noted in 70 % of male cases and 87 % of female cases) and at the time of the event (CD4 200 cell/ μ l seen in 47 % of male and 40 % of female cases).

Therefore, even though the exact mechanism for most of the factors associated with HL/LA is not well understood, it does look clear that female patients with advanced HIV disease when exposed to d4T and also to ddI are at much higher risk of developing HL/LA. In addition, it is also possible that those individuals who are non-white may be at even greater risk for this complication. A genetic mechanism for this mitochondrial dysfunction should be investigated as a matter of urgency.

7.2 Comparison between risk factors for HL and LA

As mentioned before, a few experts in the field have suggested unifying all diagnoses previously used to describe abnormalities in blood lactate metabolism into a single entity: lactic acidemia (Carr 2003; Schambelan *et al.* 2002). Although there is a clear biological plausibility for this a few issues should be considered when this approach is taken. Firstly, even when several authors have suggested that LA is the final outcome of a progressive mechanism of rising levels of blood lactate induced by NRTIs (Moyle *et al.* 2002; Powderly 2002), such a progression through increasing hyperlactatemic stages to LA has not been properly demonstrated.

In addition, even when attributable symptoms are almost always present in patients with LA, our study shows that some symptom free patients can have sustained and severe HL (10/110; 9% of our cases) and conversely, other smaller reports have established that patients with mild-moderate HL may have symptoms attributable to that condition (Hocqueloux *et al.* 2003).

Therefore, those patients with severe but asymptomatic HL can only be diagnosed through routine or random screening of blood lactate levels which is not a regular practice in most centres.

It has been proposed that mild to moderate episodes of HL can be transient and may not imply any further risk for developing serious complications (Moyle *et al.* 2002). Nevertheless, this proposal was made in reference to patients with blood lactate usually in the range of 2.5 to 5 mmol/l not in the context of patients with sustained blood lactate levels above 5 mmol/l. Six out of the 10 asymptomatic cases of HL included in our study developed further episodes of HL while continuing or after being re-challenged with combination ART including either d4T, ddI or both (d4T/ddI). This observation may suggest that those patients who develop episodes of asymptomatic severe HL are at higher risk of developing this complication at any time if they are exposed to further dideoxynucleoside analogues. None of the asymptomatic cases had a fatal outcome in our study but some of them presented characteristics associated with fatality such as current blood lactate concentration higher than 7 mmol/l (see section 7.3). Consequently, although it is difficult to speculate on the long-term implications of asymptomatic episodes of HL, given the results of our study, it may not be easy either to assume that such episodes are benign and transient elevations of blood lactate levels.

The results from our study and all previously published reports support the association between exposure to NRTIs, particularly d4T, ddI and the combination of these two, and both HL and LA. However, from table 6.13 one could assume that not all variables identified as risk factors for the combined outcome (HL/LA) behave in the same way for each outcome when considered separately. Few variables such as female gender appears strongly associated with LA (OR= 8.88; 95% CI= 2.57 – 30.64) but not necessarily with HL (OR= 1.94; 95% CI= 0.91 – 4.15). However, as with all other variables in the table 6.13, even when the effect of gender on HL does not reach statistical significance the direction of the effect is in the same direction for HL as that for LA. Therefore, considering that the study was not powered to assess

differences between each outcome considered in isolation, it is at least not possible to draw any firm conclusion on this issue.

Nevertheless, not only from the data presented in table 6.13, but also from the analysis of interactions performed (see chapter 6, section 6.2.4) it is clear that even after adjusting for all other variables, the interaction term between female gender and LA remains significant suggesting that the association between female gender and LA is quite strong. Furthermore, after adding the interaction term between female gender and LA to the multivariate model developed for the combined outcome, only the effect of female gender is affected, leading that to lose its significance while the effect of all other factors included in the model remained practically the same. This additional analysis also supports the impression of female patients being at significantly higher risk for LA but not necessarily for HL.

Nonetheless, if progression from HL to LA is part of a continuum in the natural history of the complication and if female patients are at particular higher risk of developing LA compared to male patients, one could hypothesise that episodes of HL may be somehow more severe in female patients when compared to male. Indeed, all female cases of HL included in this study were symptomatic. In addition to that, women tended to have higher blood lactate levels at the time of the event (median lactate=7.0 mmol/l; IQR= 6.01 – 7.5) than men (median lactate= 5.8 mmol/l; IQR= 5.42 – 7.35) although that difference was not statistically significant ($P= 0.075$). Furthermore, 52 % of the female cases of HL had blood lactate higher than 7 mmol/l at the time of diagnosis whereas only 20 % of male cases had levels that high ($P= 0.001$). Therefore, from the clinical and the biochemical points of view female cases of HL were in some way more severe than the episodes presented in male patients included in this study.

Data coming from a clinical trial published few years ago, shows that female patients randomised to receive either ddI monotherapy or the combination of AZT/ddI were significantly more likely to discontinue ART or to reduce dose

because of ADR than male participants (Currier *et al.* 2000). Interestingly women were also more likely to be black or Hispanic.

In conclusion, in addition to an as yet unexplained underlying special susceptibility which may be ethnicity patients with advanced HIV-induced immunosuppression who are exposed to d4T, ddI or the combination of these two NRTIs are at higher risk of developing HL/LA. Furthermore, as aging may also be associated with mitochondrial dysfunction, older patients exposed to all previously listed factors may be even at higher risk of developing such a combined outcome. Also, based on the results presented in our study, one could assume that a particular group of individuals, namely HIV-infected women when exposed to the previously mentioned factors may be at higher risk of developing LA. Furthermore, the fact that female patients are more likely to develop more severe episodes of sustained HL than male may also support the concept of progression from HL to LA.

7.3 Analysis of the outcome of the event

A few issues regarding the outcome of the event have been already discussed. However, it is important to emphasize some important issues which may have implications in the management of this complication.

7.3.1 Risk factors for mortality associated with HL/LA

Since the first cases of LA in HIV infection were published a high case fatality rate attributable to that complication was recognised. However, only one study published to date has explored factors associated with a fatal outcome. In the paper published by Falcó *et al* 12 cases were presented (Falco *et al.* 2002). The calculated case fatality rate for that series was 33 %, which is appreciably different from the rate calculated for our study (17.3 %). Nevertheless, the figure calculated by Falcó *et al.* does not differ at all from the case fatality rate we calculated in this study when only cases of LA are considered (32.7 %).

The case fatality rate calculated from the systematic review of published cases of LA we performed earlier (see chapter 4) was rather higher (47 %).

However, in our systematic review there were 90 cases of LA included, 50 % of whom were diagnosed between 1991 and 1999. The other 45 cases were diagnosed between 2000 and 2001. Interestingly, the case fatality rate among the first half of cases included in such review was 64 %, whereas the same figure calculated for the most recently diagnosed cases was 33 % (Arenas-Pinto *et al.* 2003). Similarly, the case fatality rate calculated by Mégarbane *et al.* for the review of published cases of LA, which included cases diagnosed during the 90's, was 60 % (Megarbane *et al.* 1999). The differences here are probably due to an increasing level of awareness of this complication amongst clinicians which has led to earlier diagnosis and intervention over time. Nevertheless, LA is still associated with a high mortality rate.

In contrast, it is important to mention that the case fatality rate among patients with severe and sustained HL was significantly lower than the figures presented in the previous paragraph. Only about 5 % of HL cases died as a consequence of the event. Of note, few data have been published on mortality associated with HL. For instance, in a small cohort study eight patients were diagnosed with HL (defined as 2 consecutive blood lactate > 2.1 mmol/l) and only one of them died as consequence of the event. Interestingly, the fatal case presented by Hocqueloux *et al.* went onto an episode of LA. So the authors suggested that the long-term outcome of patients with HL may not be poorer than controls (Hocqueloux *et al.* 2003). In addition, in a review on the long-term complications associated with ART, although no evidence was presented to support the statement, Powderly suggests that progression of untreated HL may lead to acidosis, which is associated with high rates of fatality (Powderly 2002). Therefore, there is not evidence of HL leading to fatal outcome unless acidosis is developed.

In this study, LA itself was identified as the strongest independent risk factor for case fatality: acidotic patients were 10 times more likely to die as

consequence of the episode when compared with non-acidotic individuals. In addition, those patients with blood lactate levels above 7 mmol/l were also at significantly higher risk of dying when compared to those with lower levels of blood lactate. Similarly, Falcó *et al.* also found that having a blood lactate level higher than 10 mmol/l was the only factor independently associated with fatality in their study (Falco *et al.* 2002). In our study the cut-off was set at 7 mmol/l as that value was the median calculated for the cases of HL/LA.

As mentioned before, it is not surprising that more severely ill patients are at higher risk of dying. However, it is important to mention that most of the previously proposed classifications for blood lactate disorders in HIV-infected patients have used a threshold of 10 mmol/l of blood lactate to either make a diagnosis of severe HL (John & Mallal 2002) or to classify a case of “lactic acidemia” regardless of the presence of symptoms (Carr 2003; Schambelan *et al.* 2002). However, in the light of results presented in our study, it may be pertinent to consider a different threshold value, as patients with blood lactate levels substantially lower than 10 mmol/l are at significantly higher risk of dying.

In addition, it is important to highlight in our study that 14 % (7/49) of the LA cases had blood lactate levels lower than 5 mmol/l at the time of diagnosis. Therefore, they would not meet the criteria as cases of lactic acidemia, according to the proposed classification. None of the seven acidotic cases who had lactate levels lower than 5 mmol/l died as consequence of the event but all of them were clearly symptomatic.

7.3.2 Further follow-up of non-fatal cases

It has been previously proposed that patients who have a history of episodes of ADR, particularly those attributable to NRTI-induced mitochondrial dysfunction, may be at higher risk of developing HL/LA (ter Hofstede *et al.* 2000; Gerard *et al.* 2000). In our study, previous ART-associated ADR and particularly those believed to be due to mitochondrial dysfunction were

significantly associated with the study outcome in the univariate analysis (OR= 3.58, 95% CI= 2.13 – 6.01 and OR= 5.87, 95% CI= 3.14 – 10.98 respectively). However, these variables were excluded from the multivariate model as it was not possible to assess the length of time between them and the current episode of HL/LA. This was because dates for such previous ADR were not collected in the study proforma. Therefore, it was not possible to exclude the possibility of the events being somehow associated with each other, i.e. they were all part of the same episode.

Nevertheless, 13 (14 %) of these 91 cases who survived their episode of HL/LA developed further episodes of HL. Interestingly, all these 13 subsequent episodes were HL and all were associated with re-exposure (or continuation of the exposure) to ART. In addition, 20 of those 91 non-fatal cases (22 %) developed other forms of mitochondrial ADR after the episode of HL/LA. So, in all, about a third of the non-fatal cases developed some form of clinically apparent mitochondrial dysfunction after being re-exposed to ART.

As the study was not powered to assess the recurrence of ADR in non-fatal cases, we cannot speculate any further on potential risk factors for subsequent episodes of HL or any other mitochondrial toxicity after a successfully managed episode of HL/LA. Nevertheless, a trend was noticed towards an increased risk for further episodes when patients were re-challenged with a d4T-containing ART combination. In the absence of any conclusive data on this issue, it may be sensible to avoid, whenever possible, to re-expose patients who have developed episodes of HL/LA to drugs likely to induce HL/LA (i.e. d4T and ddI).

7.4 Limitations of the study

Like any other retrospective study, the case-control study presented here has important limitations. Firstly, this retrospective, multi-centre study included cases diagnosed and managed in centres with different policies in the monitoring and management of patients. A few clinics included in this study

incorporate blood lactate in the set of tests routinely performed to patients. Therefore, in some but not all participant centres, it was possible to identify asymptomatic cases of severe and sustained HL. Furthermore, policies have changed in a few centres during the study period in this regard, and therefore the likelihood of identifying cases of HL has also varied over time.

In addition, even when a standardised proforma was used to collect data from both cases and controls and when most of the study proformas were either completed or audited by the principal investigator, a certain degree of reporting bias can not be excluded. The level of description of current and previous medical information in cases tended to be rather higher in some centres when compared with the available information about controls. Nevertheless, all patients' notes were searched backwards until the earliest available documented consultation and therefore, all the available information was recorded in the study proformas.

Besides, reasons for switching ART combinations were not recorded and therefore, it is not possible to assess whether or not exposure to any specific drugs was preceded by virological or immunological failure or was consequence of any ADR. Therefore, we cannot exclude the impact of high viral load or low CD4 on the effect of a given regimen. Similarly, even when previous ADR were recorded, the dates for these episodes were not. Consequently, it is difficult to analyse the occurrence of these previous ADR in the context of concurrent exposure to ART and impossible to assess how close in time these previous ADR were to the event of HL/LA.

As in many other retrospective studies, significant amounts of missing values were identified. This missing data was particularly relevant during the process of developing the multivariate model for risk factors associated with HL/LA because of the conditional analysis used. Of note, data on variables such as laboratory results and past medical history were less frequently available among controls than in cases. Therefore, because of the conditional (matched) analysis performed, when a specific variable was not reported in a

control patient its respective case was also lost for the analysis of that particular variable.

As has been mentioned before, given the lack of ethnic variability observed in the study population and because of the level of ethnic homogeneity found in most participant clinics' population, matching by centre may be an overmatching by ethnicity. Therefore, results presented in chapter 6 are likely to underestimate the association between ethnic background and HL/LA.

In addition, among all limitations classically attributed to case-control studies, information or recall bias is perhaps the less relevant to this particular one. All data were collected from clinical notes which were completed at the time of the event or at the time of the index consultation for cases, and therefore are not likely to be affected by what either clinicians or patients may recall months or even years after the event. Nevertheless, it is not possible either to exclude the possibility of cases to be a group more frequently affected by HIV-associated complications than controls. Therefore, it is possible that more clinical and laboratory data are collected in cases than in controls simply because cases were seen in clinic more often or required more attention from their doctors.

The fact that duration of d4T exposure is shorter on average among cases than the controls probably indicates that the risk of HL/LA decreases over time as has been mentioned before. Although a sort of "survivor bias" could be argued as an explanation for that, matching by calendar time results in the same effect on both cases and controls. Therefore, the reduction on the risk for the study outcome over time may be real. That is certainly a very important finding which should be investigated further.

The study population included in this case-control study represent also the largest case series of HL/LA compiled to date. Therefore, a detailed descriptive analysis of the cases included is presented. Demographic, epidemiological and clinical variables were assessed in an attempt of

producing a comprehensive picture of the group of cases included. However, it is well known that multiple comparisons certainly can generate false positive associations (Type I error). Therefore, for the study of risk factors for HL/LA, not all the variables collected were assessed in the logistic regression model. As described in chapter 5, the analysis strategy was designed before the data collection phase and was based mainly on the findings of the systematic review (chapter 4) and other previous studies available. Factors such as age, gender, severity of HIV-induced immunosuppression (both at baseline and at the time of the event), previous and concurrent ADR and detailed history of exposure to NRTI were assessed in the model developed. Other factors, although described in the previous section, were not entered in the model. However, specific statistical tests for type I errors were not performed.

CHAPTER 8

Concluding discussion

After about two decades of experience in the therapeutic management of HIV infection and after having achieved significant progress in controlling viral replication, NRTIs remain as the backbone of most ART combinations prescribed worldwide. Furthermore, NRTIs and particularly thymidine analogues (i.e. AZT and d4T) are the cornerstone of first line combination options suggested by the WHO for developing countries (World Health Organization 2003).

Therefore, most of those patients who are taking ART at the moment and the several million people more in the developing world that will start in coming years are going to be exposed to NRTIs. Of note most of the patients who are going to initiate ART in the near future are going to receive thymidine analogue based combinations. Consequently, it remains critically important to identify not only the risk factors associated with NRTI-induced ADR but also to quantify risk and to produce evidence based guidelines for the use of thymidine analogues. In addition, it is also critically important to identify an efficient approach in the management of associated complications. It will also be essential to develop and evaluate appropriate and cost-effective safety monitoring strategies considering the limited access to laboratory support in many countries of the developing world.

8.1 Effect of dideoxynucleosides on mitochondrial function

As mentioned in chapter 2, most of the NRTIs currently in clinical use, and particularly dideoxynucleosides have been shown to affect mitochondrial structure and function. Such deleterious effects on mitochondria have also been suggested as a mechanism for most NRTI-associated ADR.

8.1.1 Dideoxynucleosides as risk factor for HL/LA and PN

Both in-vitro and clinical studies have demonstrated that NRTIs can induce mitochondrial dysfunction and such mitochondrial impairment may or may not include mtDNA depletion (see chapter 2). Furthermore, most of ADR attributed to NRTIs have been explained as a consequence of mitochondrial dysfunction. In that context, both PN and HL/LA have been described as major ADR associated with d4T and ddI. Interestingly, even when ddC has also been described as a potent PN inducer (Yarchoan *et al.* 1988), not a single case of HL/LA associated with ddC has been published. However, ddC has not been prescribed to the same extent as other licensed NRTIs and therefore, a significantly smaller number of patients have been exposed to it than to other NRTIs. The last observation could be important as uncommon ADR, to be identified, require a large number of exposed individuals (Bisson *et al.* 2003). Interestingly, a few patients who were previously exposed to ddC have developed HL/LA when exposed to other NRTIs but not ddC. In fact, 19 out of the 110 cases included in the case-control study presented in chapters 5 to 7 had previous exposure to ddC as so did at least 7 of the cases included in the systematic review presented in chapter 4 (data not shown).

On the other hand, ddC exposure has been consistently associated with PN (Bisson *et al.* 2003; Fichtenbaum *et al.* 1995). In the sub-analysis of Delta presented in chapter 3 it was obvious that those patients exposed to AZT/ddC combination showed a higher incidence rate of PN when compared to patients receiving either AZT monotherapy or AZT/ddI combination. It has been mentioned before that NRTI-induced mitochondrial dysfunction may be drug and tissue specific (Vittecoq *et al.* 2002; Cherry & Wesselingh 2003). Therefore, one could assume that the capacity of a given dideoxynucleoside to induce clinically evident complications may depend not only on its ability of inducing mitochondrial dysfunction but also on other variables, which may include not only pharmacokinetic factors but also pre-existing mitochondrial dysfunctions and the effect of the HIV itself (Cherry & Wesselingh 2003; Max & Sherer 2000).

It has been proposed that HIV itself can affect the likelihood of developing mitochondrial toxicities, suggesting that those patients with more advanced HIV disease are at higher risk of developing at least some NRTI-associated ADR (Joly *et al.* 2002; Moyle 2005b). In chapter 3 some evidence is presented suggesting that patients exposed to ddC in combination with AZT in the Delta trial had a poorer clinical response to ART than those exposed to AZT/ddI combination. Therefore, the higher incidence of PN among those patients exposed to ddC may be explained not only because of the strong effect of ddC on mitochondrial function but also the effect of HIV itself or the inflammatory response to it in peripheral nerves (Keswani *et al.* 2002; Luciano *et al.* 2003). A few studies have shown that HIV-infected individuals who have never been exposed to any ART drug have a significantly lower level of mtDNA in their PBMC when compared to healthy volunteers (Cote *et al.* 2002; Shikuma *et al.* 2001). Interestingly, after a year of exposure to ART combinations those patients with baseline mtDNA depletion show a significant recovery of their mtDNA content and this was seen even among those receiving dideoxynucleoside based combinations (Gallant *et al.* 2002; Miura *et al.* 2003). The study published by Miura *et al.* has also found an association between mtDNA content and HIV disease progression which has not been confirmed by others yet.

Furthermore, not only mtDNA depletion but also significant reduction in respiratory chain complex activity has been demonstrated in treatment naïve HIV-infected individuals when compared with healthy volunteers (Miro *et al.* 2004a; Morgello *et al.* 1995). Interestingly, Miró *et al.* have found significant reduction in the activity of all respiratory chain complexes, including complex II, which is entirely encoded by nDNA. Therefore, HIV-induced mtDNA depletion may be interpreted as a marker of generalised mitochondrial damage rather than any direct effect of the virus on mtDNA replication. Interestingly, two of the cases included in the case-control study presented in chapters 5 to 7 in whom mitochondrial function tests were performed also

showed significant reduction in respiratory chain complex II in at least one cell type. These two cases were taking NRTIs at the time of the event.

In addition, several HIV proteins (e.g. TAT, vpr and HIV protease) have been proposed as capable of affecting mitochondrial function through different mechanisms (Raidel *et al.* 2002; Jacotot *et al.* 2000; Roumier *et al.* 2002; Nie *et al.* 2002). Furthermore, inflammation mediators and cytokines released in response to HIV infection or its complications (e.g. tumour necrosis factor- α or interferon- γ) have been associated with functional and morphological impairment of mitochondria in neurons and smooth muscle cells (Polla *et al.* 1996; Li *et al.* 2001; Geng *et al.* 1992). Therefore, it may be possible for HIV to affect mitochondrial function and structure either directly or indirectly and if that is the case, one could assume that the magnitude of the viral burden in a given patient may influence the likelihood of developing serious mitochondrial dysfunctions.

In summary, one could assume that HIV disease may at least modulate the effect of NRTIs and particularly dideoxynucleosides on mitochondria. Not surprisingly then, current CD4 count lower than 200 cell/ μ l was identified as an independent risk factor for HL/LA in the case-control study presented in chapters 5 to 7.

Nevertheless, it has been clearly demonstrated that NRTIs can affect mitochondrial function even in the absence of HIV infection. This has been demonstrated not only in vitro (Yamaguchi *et al.* 2002) but also in vivo. In a small study, twenty healthy male adults were exposed to either AZT/3TC or d4T/3TC for six weeks. After only two weeks of exposure these healthy individuals showed a significant reduction of mitochondrial gene expression in adipocytes and in monocytes at week six (Mallon *et al.* 2005). Interestingly Mallon *et al.* did not find any evidence of mtDNA depletion in that group of individuals.

However, given the fact that combination ART is the standard of care and because combinations in clinical use are not randomly allocated, it is difficult to analyse the effect of specific drugs on ADR and therefore, almost all NRTIs currently in clinical use have been associated with mitochondrial toxicities.

However, d4T has been consistently reported as independently associated with clinically evident mitochondrial dysfunction, particularly with HL/LA (Brinkman *et al.* 1999; Caron *et al.* 2004; Walker *et al.* 2001). The data presented in this thesis also support that association. However, when exposure to NRTIs is analysed considering the combination backbone rather than single drug exposure, the effect of d4T was found to be significant only when it was prescribed in combination with ddI. In addition, ddI was also found to be associated with HL/LA when assessed as single drug exposure (see chapter 6). The effect of ddI with or without d4T concomitant exposure should be assessed carefully, particularly because ddI has been included as a second line option for HIV treatment in developing countries by the WHO (World Health Organization 2003). Therefore, many patients who may have developed therapeutic failure or significant toxicity with first line NRTIs may be at high risk for HL/LA.

ABC has not been associated with any mitochondrial toxicity and that may be consequence of its unique phosphorylation pathway (Kakuda 2000). In addition, it has been shown that switching patients from d4T to ABC may reverse the mtDNA depletion seen at baseline not only in PBMC but also in muscle and adipose tissue as well. Furthermore, such a switching strategy can also improve the activity of respiratory chain complex I after 48 weeks (McComsey *et al.* 2005b). Although AZT has been associated with a number of ADR attributable to mitochondrial dysfunction (i.e. bone marrow suppression and LA) switching patients from d4T to AZT has also been shown to be beneficial for patients with lipodystrophy syndrome and even persistent HL (Lonergan *et al.* 2004).

On the other hand, 3TC has been shown to be a very weak inducer of mitochondrial dysfunction in vitro (Kakuda 2000) and is considered in clinical practice as a relatively safe drug. Interestingly, in the case-control study presented in chapters 5 to 7, exposure to ABC or 3TC was not associated with HL/LA as expected. Furthermore, when ART exposure is assessed using NRTIs combinations rather than single drug exposure, current treatment with AZT/3TC based combinations was found to be negatively associated with HL/LA. One could assume that being exposed to AZT may avert the study outcome by preventing patients being treated with d4T, so patients on AZT may be at lower risk of developing HL/LA because they were not taking d4T.

If 3TC is not capable of inducing significant mitochondrial dysfunction, one would expect it to have a very safe profile, at least on mitochondrial dysfunction associated ADR. Data from a large observational study have highlighted the beneficial effect of 3TC on HL. In the paper published by the Swiss Cohort in 2001 both 3TC (OR= 0.4; 95% CI= 0.2 – 0.6) and AZT exposure (OR= 0.4; 95% CI= 0.2 - 0.7) showed a protective effect for HL in the univariate analysis but they did not find any significant effect of 3TC or AZT after adjusting for other factors in the multivariate model, so the apparent beneficial effect of these drugs on protecting against mitochondrial dysfunction may be confounded by other factors (Boubaker *et al.* 2001). However, Moyle *et al.* have shown that patients exposed to some combinations including 3TC, such as 3TC/ABC or even 3TC/d4T have a significant lower risk of developing HL (defined as blood lactate > 2.5 mmol/l) than those individuals exposed to d4T/ddI (RH= 0.2; 95% CI= 0.05 – 0.85 and RH= 0.39; 95% CI= 0.23 – 0.66 respectively) (Moyle *et al.* 2002). In the case-control study presented in this thesis, 17.3 % of cases were receiving d4T/3TC based combinations at the time of the event while the proportion of controls on d4T/3TC was 21.8 % (P= 0.231). The study failed to identify any difference between cases and controls in terms of their exposure to the combination of d4T with 3TC. Although the study was powered to identify significant differences between cases and controls with regards to their exposure to d4T, the calculation was done

considering d4T as a single drug exposure and it did not take into consideration other NRTIs used in combination with d4T.

At the moment the WHO recommendations includes d4T/3TC as the preferred backbone for ART combinations to be used in resource limited settings (World Health Organization 2003). Recently presented studies in small numbers of patients have shown that reduced doses of d4T are associated with less ADR without losing potency when administered in combination with at least two other ART drugs (Milinkovic *et al.* 2005; Wolf *et al.* 2004; Siangphoe *et al.* 2004). Therefore, given the relevance of serious ADR for the long-term success of HIV/AIDS control programs, assessing the safety profile of dideoxynucleosides at reduced doses may be critically important for developing countries.

Cote has proposed a combined multi-factorial mechanism to explain mitochondrial dysfunction in HIV-infected patients exposed to NRTIs. According to the author, long-term exposure to ART may result in mitochondrial dysfunction as consequence of a triple hit mechanism where the effect of HIV and the NRTIs on mitochondrial function increases production of ROS, which completes the vicious circle amplifying the deleterious net effect on mitochondrial function (Cote 2005). Nevertheless, other possible, as yet unidentified mechanisms for NRTI-induced mitochondrial impairment may exist and therefore, more research is needed to fully clarify the underlying mechanisms for this toxicity. Unfortunately, different studies performed to date on mitochondrial function in HIV-infected individuals have used different assays and approaches making difficult if not impossible to compare results from these studies. In addition, such studies have generally involved small numbers of patients. Therefore, it is important to perform large longitudinal studies using standardised assays to produce robust information on ADR associated with mitochondrial dysfunction (Cossarizza 2003).

8.1.2 Special susceptibility

It has been suggested that environmental factors may affect the likelihood of developing any specific ADR, even in the presence of specific genetic polymorphisms shown to be clearly associated with such a complication. Factors such as co-morbidities, treatment adherence and drug-drug interactions can all affect the likelihood of developing any ADR in the presence of a specific genetic predisposition (Quirk *et al.* 2004).

Pharmacogenomic studies may generate information leading to the identification of specific genetic polymorphisms which may predispose to a particular ADR. As an example, in the HIV medicine field screening for HLA B5701 has been recently implemented in some centres as regular practice as it was demonstrated that people with that specific haplotype were at much higher risk for ABC-induced hypersensitivity reaction (Mallal *et al.* 2002). In addition, pre-prescription testing for HLA B5701 has been shown as a cost-effective intervention for prevention of ABC-induced hypersensitivity (Hughes *et al.* 2004).

Little is known about genetic predisposition to NRTI-associated mitochondrial toxic effects (Lewis 2005). However, as mentioned before, a recent study has highlighted a possible association between mitochondrial haplogroup T and an increased risk for PN in HIV-infected individuals exposed to dideoxynucleosides (Hulgan *et al.* 2005). Some additional evidence suggests that genetic polymorphisms may play a significant role in NRTI-induced mitochondrial dysfunction. Similarities between toxic mitochondrial dysfunction and inherited mitochondrial diseases and the recently published association between specific mtDNA haplogroups and PN in HIV-infected patients illustrate this point (White 2001; Hulgan *et al.* 2005). Nevertheless, assessing the impact of mtDNA variations on multifactorial diseases has been shown to be difficult. Even when initial reports may have shown promising results it has been almost always impossible to replicate original results (Samuels *et al.* 2006). Because the distribution of different mtDNA haplogroups is not uniform,

the low frequency of some haplogroups tends to increase the statistical significance of specific analyses generating false-positive results (Roff & Bentzen 1989). Therefore, proper study designs where appropriate statistical methods are used to deal with the described type I error tendency are needed to properly assess the impact of mtDNA haplogroups on the likelihood of developing NRTI-induced mitochondrial dysfunction (Samuels *et al.* 2006).

In addition, as the distribution of human genetic polymorphisms is not homogeneously distributed and significant differences, particularly in the case of mitochondrial genetics, have been found between different ethnic groups it is essential to perform pharmacogenetic studies including populations large enough to achieve robust results where different ethnic backgrounds are well represented.

Even when not powered to look at ethnicity, and despite the possible overmatching discussed in chapter 7, the case-control study presented in this thesis suggests that non-white populations may be more likely to develop HL/LA than white people. Similarly, Gerard *et al.* have shown that black women may be at higher risk of developing HL compared to white women when exposed to d4T containing combinations (Gerard *et al.* 2005).

In chapters 3 and 7 the effect of advanced HIV disease on the likelihood of developing PN and HL/LA was also discussed. Patients with low CD4 count appear to be at high risk for these complications. Therefore, one could assume that the magnitude of the problem associated with NRTI-induced mitochondrial dysfunction in the developing world could be much higher than thought, not only because d4T-based combinations are used much more often but also because among the target population the other important identified risk factors for HL/LA are over-represented (i.e. female gender and advanced HIV disease).

In addition, one can assume that a high level of suspicion among clinicians and close monitoring of patients on ART may help to identify cases at an early

stage (Stenzel & Carpenter 2000). Not surprisingly then, the calculated case fatality rate for the cases of LA presented in chapter 4 was significantly higher among cases diagnosed before the year 2000 compared to more recent cases (Arenas-Pinto *et al.* 2003). However, even when an early diagnosis is made, and even when some studies have shown improvement in mtDNA content in patients switched away from d4T (Boyd *et al.* 2004), stopping dideoxynucleoside exposure may not lead to an immediate recovery of mtDNA content. A small study has shown that previously d4T or AZT exposed patients randomised to switch therapy to ABC did not show any recovery in the content of mtDNA in PBMC at week 24 from randomisation (Hoy *et al.* 2004). In fact, patients may need at least six months after stopping ART exposure to show any significant rise in the content of mtDNA in CD8 T lymphocytes (Mussini *et al.* 2005). Interestingly, the study published by Mussini *et al.* also showed that the content of mtDNA in CD4 T lymphocytes remained unchanged one year after stopping any exposure to ART. The last observation may reflect the effect of HIV itself on the mitochondrial genome of CD4 lymphocytes.

In any case, patients exposed to dideoxynucleosides may remain susceptible to express clinical manifestations of mitochondrial dysfunction even after stopping the exposure to such drugs if mtDNA is a good marker of mitochondrial function. For instance, a fatal case of liver failure following an acute episode of LA has been published (Carr *et al.* 2001). Interestingly, the patient reported by Carr *et al.* died a few months after stopping ART.

8.2 Impact of safety issues on HIV/AIDS control programs

It has been mentioned before that the benefits of HAART combinations in the management of chronic HIV disease are endangered in the long-term by ADR (Carr 2002). Fortunately, only a few ART-associated ADR can be life threatening (e.g. LA) but even mild to moderate complications such as vomiting or diarrhoea may affect adherence to ART, which has been demonstrated to be associated with resistance (Taylor-Castillo *et al.* 2005).

Therefore, beyond the implications of ADR in clinical practice, toxicities may have a considerable impact on HIV control programs by affecting mortality, morbidity and resistance.

8.2.1 ART delivery as a public health intervention

According to UNAIDS estimates, there were just over 40,000,000 people living with HIV world-wide by the end of 2005. However, as has been highlighted almost since the beginning of the epidemic, the vast majority of those infected by HIV are living in developing countries. In fact, over 90 % of the infected population live in the developing world and almost 26 million (64 % of the entire HIV-infected population) live in Sub-Saharan countries (UNAIDS 2006).

Although the introduction of HAART combinations has had a tremendous impact in reducing mortality and morbidity associated with HIV disease in North America and Western Europe (Palella, Jr. *et al.* 1998; Mocroft *et al.* 1998), the benefits of this therapeutic approach at a population level have been very small in most developing countries. In fact, almost all (98 %) of those patients who may have died in 2005 because of HIV-related causes did so in developing countries according to UNAIDS estimates (UNAIDS 2006). Therefore, it may be obvious that access to ART drugs should be the first priority of any program addressed to control the HIV/AIDS epidemic. This is why the WHO has launched a scaling-up initiative aimed to increase significantly the number of patients on ART in developing countries. That initiative has been defined and conceived as a public health intervention and it is aimed to reduce as much as possible mortality attributable to HIV/AIDS in the developing world (World Health Organization 2003).

Interestingly, the effectiveness of HAART in resource limited settings has been shown to be not significantly different from what has been achieved in developed countries. In Thailand a “cohort” of HIV-infected patients has shown equivalent response to ART to what it may be expected in developed countries (Duncombe *et al.* 2005). However, this Thai cohort is in fact a

compilation of clinical trial participants which may not reflect the situation in real life conditions. Nevertheless, similar findings have been communicated by Médecins Sans Frontières (MSF) in an observational study on clinical outcomes of their HIV/AIDS programs in 21 resource-limited countries. The study published by Calmy *et al.* (N= 6861) shows an estimate survival of 0.82 (95 % CI= 0.81 – 0.84) at 12 months after starting a d4T/3TC/Nvp fixed drug combination therapy (intent-to-treat analysis missing equal death) (Calmy *et al.* 2006). In addition, a meta-analysis analysing data from 10 studies performed in developing countries (mainly in Africa) has shown that based on surrogate markers, efficacy of ART in resource-limited settings may be comparable to efficacy rates reported for developed countries (Ivers *et al.* 2005).

A small study performed in Venezuela (N= 96) has shown that naïve patients receiving ART as part of the national HIV/AIDS control program have achieved a significant level of response to therapy: 50 of the 67 patients tested for HIV viral load were undetectable (< 400 copies) at week 48 (Garcia 2005). However, a comparative study analysing cohorts of HIV-infected patients from both developed and developing countries has recently shown that patients starting first line HAART combinations in developing countries show a higher mortality rate than what is seen among similar patients in developed countries over the first year of therapy. Interestingly, the mortality rate fell substantially over the first months and by the second half of the first year on HAART the mortality rate in developing countries was similar to what has been recorded for developed countries (Braitstein *et al.* 2006). Similarly, the mortality rate reported by Calmy *et al.* (14.2/100 person-years; 95% CI= 13.8 – 14.5) was noted not to be constant over the follow-up period. The likelihood of dieing in the MSF cohort was significantly higher during the first three months of HAART (Calmy *et al.* 2006). Several factors may contribute to increase mortality rate in developing countries during the first months of HAART. Co-morbidities such as tuberculosis (TB) or malaria among others and of course advanced HIV disease at base line, may all affect negatively the outcome of HAART. However, it looks like that HAART-induced HIV

replication control can overcome these factors and offer long lasting benefits to patients in developing countries.

Little is known about safety issues related to ART in developing countries. Most studies on ART in these settings have been focussed on the delivery question as well as on efficacy of the intervention. However, growing concerns on ART-associated ADR and its possible impact on adherence and ultimately on resistance have been recently expressed. The Forum for Collaborative HIV Research has recently organised a workshop called Long-Term Monitoring of Treatment Related Adverse Events in the Resource Limited Setting where key issues on methodological and logistic questions were addressed (Miller & Powderly 2005). During that meeting, issues such as reduction of patients' confidence on ART due to real and assumed ADR and the implications for adherence and therapeutic failure as a result of resistance were highlighted as major problems for HIV/AIDS control programs (Katabira 2005).

8.2.2 An opportunity for pharmacoepidemiological studies

A number of reports have been recently presented showing data on monitoring ADR in developing countries both in the context of clinical trials and surveillance as a component of national HIV/AIDS control programs. The clinical trial cohort in Thailand has recently shown that grade III/IV laboratory abnormalities (mostly abnormal LFT, hyperlipidemia, anaemia and thrombocytopenia) are frequently seen among their study population (Nuesch *et al.* 2006). Interestingly, even though AZT, ddI and d4T were part of the therapeutic options in use, blood lactate levels were not reported in the last study. On the other hand, centres involved in ART delivery as part of the South African HIV/AIDS control program have reported a rate of switching patients away from d4T of 15 per 1000 person-years because of HL/LA and 17 per 1000 person-years because of PN (Boulle *et al.* 2006). Similarly, data from a Ugandan study have shown that 21 % of over a thousand patients who started their first line ART combination were switched to a different combination because of toxicity. Of those Ugandan patients with ADR 82 %

were switched away from d4T (Forna *et al.* 2006). Likewise, it has been showed that in DART, one of the largest clinical trials in Sub-Saharan Africa, the incidence of grade IV anaemia is significantly greater than what has been reported in developed countries (Ssali *et al.* 2005). However, it was also noted in DART that the vast majority of patients showed a significant rise in their haemoglobin levels by week 24 of treatment. Base-line low haemoglobin level, due to nutritional factors, helminth infections and even malaria, may explain the findings in DART. On the contrary, the reported rate for NRTI-associated ADR in the MSF study was rather lower. In the safety sub-study reported by Calmy *et al.* (N= 655) 30 out of 52 patients followed for 12 months had to discontinue ART because of severe toxicity (grade III or IV). Interestingly, only nine of the 30 severe episodes of ADR reported by MSF in their cohort were attributable to d4T (i.e. PN and Lipodystrophy) (Calmy *et al.* 2006).

According to WHO/UNAIDS data, by December 2005 there were more than one million people receiving ART regularly in the developing world (Estimated number of individuals on ART = 1,330,000). 61 % of those patients were being treated in Sub-Saharan Africa whereas 24 % were in Latin America and the Caribbean (WHO/UNAIDS 2006). Most of the control programs including ART delivery in Sub-Saharan African countries have been implemented over the past few years while several programs in Latin American and Caribbean countries have been in place for over a decade now. In any case, it is obvious that the expansion of accessibility to ART in the developing world is giving us the opportunity to offer ART to many more people than ever before. Furthermore, because of the epidemiological characteristics of the HIV/AIDS epidemic in resource limited settings, we are now treating many more women and children. Because women and children are usually excluded from clinical trials and because the HIV-infected population in western developed countries included mainly men, the information we have produced through clinical trials and post-marketing surveillance over the past years on safety issues do not provide sufficient information in these populations groups.

Taken all together, one could easily assume that information becoming available from the developing world would be critically important in expanding our understanding of ART-associated ADR. Nevertheless, given the limited availability in most developing countries for regular follow-up of patients on ART and laboratory support, specifically designed studies in these settings may be needed to generate relevant information not likely to be obtained from regular monitoring. Furthermore, as surveillance systems in developing countries have been traditionally weak and in most cases inconsistently applied, relying on such traditional surveillance approaches for safety issues associated with ART may not be the most appropriate approach. Here is where pharmacoepidemiological studies may have a critical role in generating clean and reliable data. Such studies range in a hierarchy from spontaneous case reports through case-control studies and observational cohort studies and clinical trials. All can generate valid and useful information. Nevertheless, novel approaches such as sentinel surveillance systems may be more appropriate options. The pharmacovigilance program in South Africa combines a novel electronic spontaneous reporting system which is complemented with a sentinel surveillance scheme to investigate signals generated in the reporting system (Banoo 2005).

Given the sustained growth in the number of individuals on ART in developing countries and because of the lack of laboratory support strong enough for monitoring patients on ART in the same way that is done in developed countries, generating robust information for a more efficient diagnosis and management of ART-associated ADR may be a major challenge. ADR have been shown as major contributors for intentional non-adherence to ART both in developed and developing countries (Bonolo *et al.* 2005; Mocroft *et al.* 2001) and adherence is certainly the most important strategy to avoid resistance. In the context of limited options for ART in the developing world, resistance is a major threat for HIV/AIDS control programs. Dealing with a rapid requirement for second line options will make programs too expensive for countries with very limited funds (Katabira 2005; Kent *et al.* 2003).

Finally, because of the limited number of drugs developing countries can afford to include in their programs, a better understanding of factors associated with ADR attributable to NRTIs, particularly AZT, d4T and even ddI, is essential to protect such programs.

8.3 Retrospective and observational data: valid sources of information

Randomised clinical trials are likely to produce clean data but their results are usually “artificial” as generally only highly selected individuals are allowed to take part in these studies. Patients with more advanced or complicated disease, those with co-morbidities, patients exposed to other therapeutic interventions and very often women and children are generally excluded from clinical trials. Therefore, beyond other disadvantages such as duration of follow-up, logistic issues and costs, most clinical trials are not likely to produce sufficient information on safety questions (Ioannidis & Lau 2001; Strom 2000).

On the other hand, observational studies can offer the opportunity to compile robust information on specific groups such as women or intravenous drug users as well as other populations generally not included in clinical trials. In addition, the effect of duration of the exposure to any ART drug can also be evaluated in prospective data generated through cohort studies (Bisson *et al.* 2003). In addition, nested case-control studies have been performed to assess many questions from behavioural issues to genetic susceptibility to HIV infection (Shrestha *et al.* 2006; Todd *et al.* 2006). Therefore, such an approach may be also valid to assess specific questions on ADR.

Nevertheless, to be regularly recorded any medical condition must be previously defined and included in the template used for data capture in any established cohort (Bisson *et al.* 2003). However, one of the major problems in studying ADR is the lack of universally accepted definitions for some of them (Carr 2002). Discrepancies in definition of ADR have been suggested to be even more notorious for complex conditions such as lipodystrophy

syndrome or metabolic complications, but even ADR known for longer such as PN may be difficult to define and classify (Moore *et al.* 2000).

In the case-control study on LA/HL presented in this thesis (chapters 5 – 7) it was demonstrated that patients with sustained and severe HL may be asymptomatic and therefore they would not be considered as cases of lactic acidemia if the classification system proposed by the USA-IAS panel was used (Schambelan *et al.* 2002). Similarly, cases of LA were found to have blood lactate levels lower than 5 mmol/l and consequently following the same classification system, they would not be diagnosed either with lactic acidemia as arterial pH is not included in the diagnosis criteria (see chapter 7, section 7.1.1). Therefore, retrospective data can help in retrieving information which may help in properly defining ADR and such definitions may be used to generate prospective data subsequently.

8.3.1 Limitations of retrospective studies in the current environment in the UK

Many disadvantages of retrospective studies have been already discussed in this thesis (see chapters 5, section 5.1 and 7, section 7.4). Nevertheless other factors may be relevant in the process of generating valid data from retrospective studies.

In the UK the Data Protection Act 1998 has been conceived to protect confidentiality and ethics committees have been very active in protecting patients' rights. For instance, it was agreed for the case-control study presented in chapters 5 to 7, all study participants had to be recruited and consented by their regular doctor. This is apparently a common practice in many epidemiological research projects. Ward *et al.* in an educational debate published in the BMJ recently have questioned the implications of such a practice. For a national case-control study on Creutzfeldt-Jakob disease the authors tried to consent 2,804 controls through their general practitioners but only 37 % of them actually replied to the invitation. At the end, the overall

response rate for controls was 16 % (Ward *et al.* 2004). It is clear that such an extremely low control recruitment rate seriously compromises the validity of any analysis to be performed and therefore limits the possibility of conducting valid community based epidemiological research in this country.

For the case-control study presented in this thesis we recruited most of the controls we planned to because HIV-infected patients are a “captive” population. Patients are seen regularly by their doctor in order to get not only their treatment but also the essential monitoring all patients know is needed. Therefore, even when the recruitment rate through the initial letter system was relatively low (see chapter 5, section 5.5.4), it was possible to approach all selected controls during their regular appointments. Nevertheless, even though it was possible to consent most of the originally selected controls for the study, the process took much longer than we anticipated, generating extra costs and delaying the start of the study.

Centres in the UK were not the only ones requested to seek written consent from every participant in the case-control study on HL/LA. However, the system used in the UK was certainly the most complicated. On the other hand, centres from countries where generic consent for retrospective research is in place (e.g. the US, Switzerland, Spain, and the Netherlands) managed to complete the data collection exercise in a shorter period of time and at a lower cost.

Protecting patients' rights is certainly important. Nevertheless, patients are or at least should be the ultimate beneficiaries of medical research and therefore, any obstacle in the process of performing pertinent and valid research can ultimately affect the rights of patients. Some evidence suggests that patients affected by chronic diseases such as cancer, may be more motivated than the general population to support research in that particular field. For instance a large cross-sectional survey performed to assess patients' views on the use of personal identifiable medical data for research found that over 80 % of the

2,955 interviewed patients did not consider the confidential use of personal information as an invasion of privacy (Barrett *et al.* 2006).

Alternative approaches for retrospective epidemiological research, where no direct contact between researchers and patients is needed and where only information already generated and recorded in medical notes are going to be used, may be designed without affecting significantly either patients' confidentiality nor patients' privacy. Generic consent is for instance a strategy which has been used successfully in other countries. In addition, assessing the views of HIV-infected community on this issue may be helpful in generating acceptable procedures for inclusion of patients in epidemiological research projects.

8.4 Recommendations

Because most of the patients currently exposed or likely to be exposed in the near future to NRTIs traditionally associated with mitochondrial toxicity are from developing countries, pharmacoepidemiological studies in such settings are urgently needed. As has been mentioned before, there is limited information regarding the nature, frequency and predictors of ADR in HIV-infected populations from resource limited countries (Kent *et al.* 2003). Furthermore, because of the epidemiological patterns of the HIV epidemic in the developed world there is little experience in management of ADR in female patients and children which represent a significant proportion of the HIV-infected population from the developing world. Therefore, studies on safety issues related to ART should preferentially assess specific questions related to these particular groups.

Relying on regular surveillance systems in developing countries may not be the most appropriate strategy to identify cases of ART-induced ADR. Active monitoring strategies for ADR may be a more suitable intervention in such settings. In fact, sentinel surveillance systems may generate accurate and clean data on specific issues such as ADR.

Therefore, case-control studies nested on well designed sentinel surveillance systems, which in fact will constitute cohorts of HIV-infected individuals, may offer the most appropriate source of information regarding specific questions on ART safety.

In addition, it may be worth assessing genetic predisposition for NRTI-associated ADR since these complications can have serious consequences not only for individual patients exposed to such drugs but also on the effectiveness and stability of HIV/AIDS control programs in the developing world. Therefore, pharmacogenomic studies, particularly assessing mitochondrial genetic polymorphisms may offer important information to guide a better ART selection in specific population groups to avoid certain ADR. This approach may be pertinent also because of the lack of laboratory support in most developing countries for monitoring patients during ART.

Finally, in the light of the results presented in this thesis, the definition of lactic acidemia may need to be reviewed. Producing clear and unambiguous case definitions for known ADR related to ART is critically important for them to be recorded systematically. Universally accepted case definitions are essential for comparative analysis between patients seen in different settings. Both prospective and retrospective data may be useful in assembling more appropriate case definitions for ADR.

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Appendix 1

Proforma and patient information sheets used for the
Case-control study on HL/LA



**CASE-CONTROL STUDY ON
LACTIC ACIDOSIS and SEVERE HYPERLACTATAEMIA
IN HIV-INFECTED PATIENTS**



ID Number (id)

First fields: Clinic code Central field: 0. Case 1-2. controls

Date of LA or HL Diagnosis (datedx)

Date of the episode or outpatient control appointment
11/11/17. NA

Date of admission (datead)

11/11/17. NA 11/11/19. NI

Diagnosis of admission (dxadm)

- | | | |
|-----------|-------------|----------------|
| 1. LA | 2. HL | 3. Sepsis |
| 4. Cancer | 5. Other AE | 6. Other _____ |
| 7. NA | | |

Clinic or HIV unit caring for patient (clinic)

- | | | |
|---------|--------------|--------------|
| 01. MMC | 02. KSC | 03. NMS |
| 04. BRI | 05. PET | 06. CH&W |
| 07. SM | 08. MAN | 09. ARG |
| 10. SYD | 11. PER | 12. VAN |
| 13. BAR | 14. ZUR | 15. AMS |
| 16. SL | 17. Other UK | 18. Other OS |

Hospital where the patient was admitted _____ (hospital)

Date of birth (datebirt)

Sex ☐ (sex)
0. Female 1. Male 9. NI

Pregnancy ☐ (pregn)
0. No 1. Yes 7. NA

Menopause ☐ (menop)
0. No 1. Yes 7. NA

Country of origin ☐ (country)

- | | | |
|------------------|----------------------|-----------------|
| 1. UK | 2. Other EU | 3. US/Canada |
| 4. Europe non-EU | 5. Africa Sub-Sahara | 6. Africa other |
| 7. Australia | 8. Other _____ | |
| 9. NI | | |

Ethnic group..... ☐ (race)

- | | | |
|-------------------|-------------------|--------------------|
| 1. White European | 2. White American | 3. Black Caribbean |
| 4. Black African | 5. Black American | 6. Asian |
| 7. Hispanic | 8. Other _____ | 9. NI |

Date of first HIV positive result / / (datehiv)
11/11/19. NI

Mode of HIV infection acquisition ☐ (mode)

- | | |
|-----------------------------------|-----------------|
| 1. Homosexual | 2. Heterosexual |
| 3. IDU | 4. Vertical |
| 5. Transfusion or needle accident | 9. NI |

AIDS diagnosis ☐ (aids)

0. No 1. Yes
Criteria: CDC class C

Previous AIDS defining illness

- | | | | |
|----------------------------------|--------------------------|---|--------------------------|
| 01. Oesoph/bronch candidiasis . | <input type="checkbox"/> | 02. Coccidioidomycosis | <input type="checkbox"/> |
| 03. Cryptococcosis | <input type="checkbox"/> | 04. Cryptosporidiosis | <input type="checkbox"/> |
| 05. CMV | <input type="checkbox"/> | 06. Encephalopathy HIV ... | <input type="checkbox"/> |
| 07. Herpes virus infection | <input type="checkbox"/> | 08. Histoplasmosis | <input type="checkbox"/> |
| 09. Isosporiasis | <input type="checkbox"/> | 10. Kaposi's sarcoma | <input type="checkbox"/> |
| 11. Lymphoma | <input type="checkbox"/> | 12. <i>M.avium</i> or <i>M.kansasii</i> | <input type="checkbox"/> |
| 13. TB | <input type="checkbox"/> | 14. PCP | <input type="checkbox"/> |
| 15. PML | <input type="checkbox"/> | 16. Recurrent Salmonella . | <input type="checkbox"/> |
| 17. Cerebral Toxoplasmosis | <input type="checkbox"/> | 18. Wasting Syndrome | <input type="checkbox"/> |
| 19. Recurrent Pneumonia | <input type="checkbox"/> | 97. NA | <input type="checkbox"/> |

Variable: aidsdef# 0. No 1. Yes

Nadir CD4 count (nadcd4)
999. NI

Peak Viral Load (peakvl)
9999999. NI
(both nadir CD4 count and peak viral load, prior to therapy)

Previous medical history

Metabolic

11. DM..... ☐
 12. Glycogen storage .. ☐
 disease

Hepatic

21. Chronic viral hep B.. ☐
 22. Chronic viral hep C. ☐
 23. Biliary disease ☐
 24. Other hepatic dis. ... ☐

Neurological

41. Seizures ☐

Myopathies:

61. Myopathy ☐

Negative

97. NA ☐
 99. NI ☐

Drug use

31. Ethanol ☐
 32. IV ☐
 33. Biguanides ☐
 (Metformin)
 34. Salicylates ☐
 35. Carbamazepine ☐
 36. Isoniazid ☐
 37. Fructose ☐
 38. Sorbitol ☐
 39. Other ☐

Malignancies:

51. Cancer (non-HIV related) ☐

Other

71. Specify ☐

Variables: medhist#

0. No

1. Yes

ART history (starting for the regimen at the LA event time)

Regimen	Drugs	Start date	Stop date
1			
2			
3			
4			
5			
6			
7			

01. Abacavir (abc)

04. Stavudine (d4T)

07. Delavirdine (dvn)

10. Amprenavir (apv)

13. Ritonavir (rtv)

16. Tenofovir (tfv)

97. NA

02. Didanosine (ddl)

05. Zalcitabine (ddC)

08. Efavirenz (efv)

11. Indinavir (idv)

14. Saquinavir (sqv)

17. Hydroxyurea (hyd)

99. NI

03. Lamivudine (3tc)

06. Zidovudine (zdv)

09. Nevirapine (nvp)

12. Nelfinavir (nfv)

15. Lopinavir (lpv)

18. Other

Variables:

Start date: drugsta

Stop date: drugsto

Previous Adverse effects related to ART

1. Peripheral neuropathy	<input type="checkbox"/>	2. Myopathy	<input type="checkbox"/>
3. Pancreatitis	<input type="checkbox"/>	4. Myelosuppression	<input type="checkbox"/>
5. Body shape changes	<input type="checkbox"/>	6. Mild-mod hypercholesterolemia	<input type="checkbox"/>
7. Severe hypercholesterolemia	<input type="checkbox"/>	8. Mild-mod hypertriglyceridemia	<input type="checkbox"/>
9. Severe hypertriglyceridemia	<input type="checkbox"/>	10. Diabetes	<input type="checkbox"/>
11. Hepatopathy	<input type="checkbox"/>	12. Osteopenia/osteoporosis	<input type="checkbox"/>
13. Other:	<input type="checkbox"/>	97. None reported	<input type="checkbox"/>

Variables: preadv# 0. No 1. Yes
Criteria: Hypercholesterolaemia: mild-mod < 6.5 mmol/l severe > 6.5 mmol/l
Hypertriglyceridaemia: mild-mod < 5 mmol/l severe > 5 mmol/l

Previous laboratory abnormalities

01. Lactate <input type="checkbox"/>	02. LFT <input type="checkbox"/>	03. Amylase <input type="checkbox"/>
04. Glucose <input type="checkbox"/>	05. pH <input type="checkbox"/>	06. Anion gap <input type="checkbox"/>
07. CK <input type="checkbox"/>	08. TC <input type="checkbox"/>	09. TG's <input type="checkbox"/>
10. Albumin <input type="checkbox"/>	11. Non <input type="checkbox"/>	12. Other <input type="checkbox"/>

Variables: prevlab# 0. No 1. Over normal 2 Under normal

Normal Weight (weighnor)	Current Weight (weighcur)	Height (height)	Normal BMI (bminor)	Current BMI (bmcur)

Normal Weight: at the last asymptomatic control
Current Weight: at the time of the LA or inclusion

97. NA

99.NI

Weight problems at the time of the inclusion ☐ (weight)

- | | |
|---|--|
| 1. Overweight (BMI > 25 Kg/m ²) | 2. Underweight (BMI < 18.5 Kg/m ²) |
| 3. Normal weight (BMI 18.5 – 24.9 Kg/m ²) | 9. NI |

Clinical symptoms at the LA event time

01. Abdominal pain	<input type="checkbox"/>	02. Nausea	<input type="checkbox"/>
03. Vomiting	<input type="checkbox"/>	04. Anorexia	<input type="checkbox"/>
05. Jaundice	<input type="checkbox"/>	06. Fatigue	<input type="checkbox"/>
07. Weight loss	<input type="checkbox"/>	08. CNS impairment	<input type="checkbox"/>
09. Dyspnoea	<input type="checkbox"/>	10. Tachypnoea	<input type="checkbox"/>
11. Weakness	<input type="checkbox"/>	12. None	<input type="checkbox"/>
13. Other	<input type="checkbox"/>	97. NA	<input type="checkbox"/>

Variables: symp# 0. No 1. Yes

Other investigations/imaging

Abdominal ultrasound	<input type="checkbox"/>	(abultras)
Abdominal CT Scan	<input type="checkbox"/>	(abctscan)
DEXA	<input type="checkbox"/>	(dexa)
Liver biopsy	<input type="checkbox"/>	(liverbx)
Muscle biopsy	<input type="checkbox"/>	(musclebx)
Other biopsy	<input type="checkbox"/>	(otherbx)
Other Imaging	<input type="checkbox"/>	(otherim)

0. Normal

1. Abnormal

2. Not done

Results of laboratory tests

Varib.	Test	Admission	Peak or nadir	Normal values
(lact)	Lactate			
(ph)	pH			
(hco3)	HCO ₃			
(agap)	Anion-gap			
(pco2)	PCO ₂			
(po2)	PO ₂			
(wbcc)	WBCC			
(neutro)	Neutrophils			
(hb)	Hb			
(cd4)	CD4			
(vl)	VL			

All variables:

Admission: variable

Peak or nadir value: variable

99999: NI

VL:

49: Undetect

Varib.	Test	Result	Normal Values	Abnormal	Times over NV (varout)	Peak or Nadir
(alkphos)	Alka. Phosphat					
(alt)	ALT					
(ast)	AST					
(lip)	Lipase					
(amyl)	Amylase					
(ck)	CK					
(gluc)	Glucose					
(cholest)	Total cholesterol					
(trigly)	Triglycerides					
(alb)	Albumin					
(creat)	Creatinine					
(bun)	Urea					

Abnormal = Is the result in the normal values range (variable name)

0. No

1. Yes

9. NI

Times over of the normal values range

97. NA

Markers of hepatic infections

Marker	Status	Date	Result
HBsAg (hbsag)			
HBeAg (hbeag)			
Anti-HBc (antihbc)			
Anti-HBs (antihbc)			
Anti-HBe (antihbe)			
Anti-HCV (antihcv)			
HCV-RNA (hcvrn)			

Status: variables

0. Neg

1. Pos

2. Not done

9. NI

Result: variabler

Results of the other studies

Study	Description	Conclusion
Liver ultrasound (livultr)		
CT scan (ctscan)		
Dexa (dexa)		
Other imaging (otherim)		
Liver biopsy (livbx)		
Muscle biopsy (mucbx)		
Autopsy (autop)		
Other histology		

Liver biopsy: predominant feature ☐ ☐ (livbxla)

- | | |
|------------------------------|-----------------------------|
| 01. Microvesicular steatosis | 02. Macrovesicular steat. |
| 03. Focal steatosis | 04. Mixed steatosis pattern |
| 05. Inflammation | 06. Necrosis |
| 07. Fibrosis | 08. Cholestasis |
| 09. Normal | 97. NA |

Liver ultrasound: predominant feature ☐ (livusla)

- | | |
|-----------------|-----------------------|
| 1. Hepatomegaly | 2. Fatty infiltration |
| 3. Cholestasis | 4. Other _____ |
| 5. Normal | 7. NA |

Liver CT Scan: predominant feature ☐ (livctla)

- | | |
|-----------------|-----------------------|
| 1. Hepatomegaly | 2. Fatty infiltration |
| 3. Cholestasis | 4. Other _____ |
| 5. Normal | 7. NA |

Tissue used for mitochondrial studies ☐ (mitused)

- | | | |
|----------|-----------|----------|
| 0. PBMC | 1. Muscle | 2. Liver |
| 3. Other | 7. NA | |

Mitochondrial dysfunction evidence

- | | | | |
|--------------------------|--------------------------|----------------------------------|--------------------------|
| 1. Enlargement | <input type="checkbox"/> | 2. Shape alteration | <input type="checkbox"/> |
| 3. Density increased ... | <input type="checkbox"/> | 4. Respiratory chain dysfunction | <input type="checkbox"/> |
| 5. Reduction of mtDNA | <input type="checkbox"/> | 6. MtDNA Deletion | <input type="checkbox"/> |
| 7. None | <input type="checkbox"/> | 8. Other _____ | <input type="checkbox"/> |
| 97. NA | <input type="checkbox"/> | | |

Variables: mitab# 0. No 1. Yes 2. Not done

Mitochondrial studies

Study	Status	Result	% of control
MRC Complex I (comp1)			
MRC Complex II (comp2)			
MRC Complex III (comp3)			
MRC Complex IV (comp4)			
MRC Complex V (comp5)			
mtDNA/nDNA ratio (dnarat)			

Variables: Status: namest 0. Not done 1. Done
Result: namers 0. Normal 1. Low 2. High 7. NA
% Control: namepor

Concurrent OI or malignancy

01. Oesoph/bronch candidiasis	<input type="checkbox"/>	02. Coccidiomycosis	<input type="checkbox"/>
03. Cryptococcosis	<input type="checkbox"/>	04. Cryptosporidiosis	<input type="checkbox"/>
05. CMV	<input type="checkbox"/>	06. Encephalopathy HIV	<input type="checkbox"/>
07. Herpes virus infection	<input type="checkbox"/>	08. Histoplasmosis	<input type="checkbox"/>
09. Isosporiasis	<input type="checkbox"/>	10. Kaposi's sarcoma	<input type="checkbox"/>
11. Lymphoma	<input type="checkbox"/>	12. <i>M.avium</i> or <i>M.kansasii</i>	<input type="checkbox"/>
13. TB	<input type="checkbox"/>	14. PCP	<input type="checkbox"/>
15. PML	<input type="checkbox"/>	16. Recurrent Salmonella	<input type="checkbox"/>
17. Cerebral Toxoplasmosis	<input type="checkbox"/>	18. Wasting Syndrome	<input type="checkbox"/>
19. Recurrent Pneumonia	<input type="checkbox"/>	97. NA	<input type="checkbox"/>

Variable: oi# 0. No

1. Yes

Other important drugs taken at the LA event time

Drug	Start date	Stop date

- | | | |
|-------------------------------------|-------------------------------|-------------------------------|
| 01. TMP/SMZ (tmprsmz) | 02. Fluconazole (flucon) | 03. Itraconazole (itracon) |
| 04. Ketoconazole (ketocon) | 05. Isoniazid (inh) | 06. Rifampicin (rifamp) |
| 07. Pyrimethamine (pyrimeth) | 08. Acyclovir (acyclov) | 09. Gancyclovir (gancyclo) |
| 10. Foscarnet (foscar) | 11. Steroids (steroids) | 12. Bleomycin (bleomy) |
| 13. Vinblastine (bleomyc) | 14. Doxorubicin (doxor) | 15. Lipo. Doxorub. (lipodox) |
| 16. Liposomal Daunorubicin (lipdau) | 17. Vincristine (vincr) | 18. Interferon α (inf) |
| 19. Thalidomide (thali) | 20. Other cytotoxic (cytotox) | 21. Contraceptive pill (aco) |
| 22. Other drug _____ | (otherdr1) | 97. NA |

Variables:

Sart: drugsta

Stop: drugsto

Concurrent Adverse events at the time of LA event

1. Peripheral neuropathy	<input type="checkbox"/>	2. Myopathy	<input type="checkbox"/>
3. Pancreatitis	<input type="checkbox"/>	4. Myelosuppression	<input type="checkbox"/>
5. Body shape changes	<input type="checkbox"/>	6. Mild-mod hypercholesterolemia	<input type="checkbox"/>
7. Severe hypercholesterolemia	<input type="checkbox"/>	8. Mild-mod hypertriglyceridemia	<input type="checkbox"/>
9. Severe hypertriglyceridemia	<input type="checkbox"/>	10. Diabetes	<input type="checkbox"/>
11. Hepatopathy	<input type="checkbox"/>	12. Osteopenia/osteoporosis	<input type="checkbox"/>
13. Other: _____	<input type="checkbox"/>	97. None reported	<input type="checkbox"/>

Variables: preadv#

0. No

1. Yes

Criteria: Hypercholesterolaemia:

mild-mod < 6.5 mmol/l

severe > 6.5 mmol/l

Hypertriglyceridaemia

mild-mod < 5 mmol/l

severe > 5 mmol/l

ART was stopped at LA event time (as a medical indication) ☐ (stoparv)
 0. No 1. Yes 7. NA

Fatal outcome ☐ (death)
 0. No 1. Yes 7. NA

Date of death (timede)
 11/11/17. NA

Date of ICU admission (icudate)
 11/11/17. NA

Management

1. Riboflavin	<input type="checkbox"/>	2. IV bicarbonate	<input type="checkbox"/>
3. IVABs	<input type="checkbox"/>	4. Ent/parenteral feeding	<input type="checkbox"/>
5. Dialysis	<input type="checkbox"/>	6. Ventilation	<input type="checkbox"/>
7. Thiamine	<input type="checkbox"/>	8. Other	<input type="checkbox"/>
9. None	<input type="checkbox"/>	97. NA	<input type="checkbox"/>

Variables: manag# 0. No 1. Yes 7. NA

ART was restarted after LA event ☐ (restart)
 0. No 1. Yes 7. NA 9. NI

ART regimen restarted ☐ (drrestart)
 1. Including NRTI 2. Not including NRTI
 7. NA 9. NI

ART regimen started after LA event

Regimen	Drugs	Start date	Stop date
1			
2			
3			
4			

01. Abacavir (abc)	02. Didanosine (ddl)	03. Lamivudine (3tc)
04. Stavudine (d4T)	05. Zalcitabine (ddC)	06. Zidovudine (zdv)
07. Delavirdine (dvn)	08. Efavirenz (efv)	09. Nevirapine (nvp)
10. Amprenavir (apv)	11. Indinavir (idv)	12. Nelfinavir (nfv)
13. Ritonavir (rtv)	14. Saquinavir (sqv)	15. Hydroxyurea (hyd)
16. Tenofovir (tfv)	17. Other	97. NA
99. NI		

Variables: Start date: ndruga Stop date: ndruga

HIV markers after the event

	First Control		Last Control	
	Result	Date	Result	Date
CD4				
VL				

Variables: CD4= cdfirstime / cdlastime / cdfirdate / cdlasdate / 997: NA
 VL= vfirstime / vlastime / vfirdate / vlasdate / 999997:NA

Time of follow-up after LA event (survival)
 1-24. months 99. NI 97. NA

Further LA event (furthla)
 0. No 1. LA 2. HL
 7. NA 9. NI

Other Adverse event after ART restarted

- | | | | |
|------------------------------------|----------------------|----------------------------------|----------------------|
| 1. Peripheral neuropathy | <input type="text"/> | 2. Myopathy | <input type="text"/> |
| 3. Pancreatitis | <input type="text"/> | 4. Myelosuppresion | <input type="text"/> |
| 5. Body shape changes | <input type="text"/> | 6. Mild-mod hypercholesterolem | <input type="text"/> |
| 7. Severe hypercholesterolemia .. | <input type="text"/> | 8. Mild-mod hypertriglyceridemia | <input type="text"/> |
| 9. Severe hypertriglyceridemia ... | <input type="text"/> | 10. Diabetes..... | <input type="text"/> |
| 11. Other: | <input type="text"/> | 97. None reported | <input type="text"/> |

Variables: furthadv# 0. No 1. Yes
 Criteria: Hypercholesterolaemia: mild-mod < 6.5 mmol/l severe > 6.5 mmol/l
 Hypertriglyceridaemia mild-mod < 5 mmol/l severe > 5 mmol/l

Further AIDS defining illness

- | | | | |
|----------------------------------|----------------------|---|----------------------|
| 01. Oesoph/bronch candidiasis | <input type="text"/> | 02. Coccidiomycosis | <input type="text"/> |
| 03. Cryptococcosis | <input type="text"/> | 04. Cryptosporidiosis | <input type="text"/> |
| 05. CMV | <input type="text"/> | 06. Encephalopathy HIV | <input type="text"/> |
| 07. Herpes virus infection | <input type="text"/> | 08. Histoplasmosis | <input type="text"/> |
| 09. Isosporiasis | <input type="text"/> | 10. Kaposi's sarcoma | <input type="text"/> |
| 11. Lymphoma | <input type="text"/> | 12. <i>M.avium</i> or <i>M.kansasii</i> ... | <input type="text"/> |
| 13. TB | <input type="text"/> | 14. PCP | <input type="text"/> |
| 15. PML | <input type="text"/> | 16. Recurrent Salmonella ... | <input type="text"/> |
| 17. Cerebral Toxoplasmosis | <input type="text"/> | 18. Wasting Syndrome | <input type="text"/> |
| 19. Recurrent Pneumonia | <input type="text"/> | 97. NA | <input type="text"/> |

Variable: furoi# 0. No 1. Yes

Form completed by: _____

Date: _____

**Severe Hyperlactataemia and Lactic Acidosis in
patients taking anti-retroviral therapy**
Patient-information sheet

You are being invited to take part in a research study. Before you decide it is important for you to understand why the research is being done and what it will involve. Please take time to read the following information carefully and discuss it with others if you wish. Ask us if there is anything that is not clear or if you would like more information. Take time to decide whether or not you wish to take part.

Thank you for reading this.

- What is the purpose of the study?

The build up of lactic acid in the blood causing the blood to be more acid (lactic acidosis) is an uncommon side effect of antiretroviral therapy (ART) but it can be fatal. However, a build up of lactic acid, which is not sufficient to make the blood more acid (severe hyperlactataemia) is believed to occur before patients develop lactic acidosis. If treatment is stopped early enough in patients who have hyperlactataemia, then usually they do not progress to severe lactic acidosis.

Because these two conditions are uncommon, information about the factors that might put patients at risk of them is lacking. The main aim of the study is to identify things that might increase the risk of developing both these conditions. We are also interested in the things that also might make some patients develop a fatal illness.

One way of achieving these aims is to compare people who have had these complications (cases) with people who have not but have received antiretroviral ART in the same way (controls).

Because the conditions are uncommon this study is going to be carried out in a large number of centres in the United Kingdom and also in other countries in Europe, the Americas and Australia.

- Why have I been chosen?

According to your regular doctor or the clinic database, you have either had an episode of lactic acidosis or considerably raised lactate level in the blood (hyperlactataemia) in the past. We are interested in collecting information about you and your condition.

- What type of information are we interested in?

We are planning to collect information about your HIV medical history, including the date of your first HIV positive test, how you acquired HIV, the type of treatment that you have been given, other illnesses that you might have suffered and other treatments for other medical conditions. We would also like to know whether you have had other side effects from ART and any previous abnormal laboratory tests.

In addition, we would like to collect information about any HIV related symptoms that you have and laboratory tests and other investigations such as the CD4 count and level of virus and any medical care during the episode of the lactic acidosis or hyperlactaemia.

Finally, some general information such as your date of birth, gender, and country of origin or ethnic group would also be collected.

Many of these factors might be important in determining whether people develop lactic acidosis or hyperlactataemia.

- What will happen to me if I take part?

The study will only use information extracted from your clinical records. No direct contact will occur between you and the researchers. This is what is called a 'retrospective case controlled study'. Again, we are planning to collect information from patients who have suffered from these complications (cases) and compare them with randomly selected patients who have never had these complications (controls).

There are no risks of taking part in this study.

- Will my taking part in this study be confidential?

All information obtained from the clinical reports will be strictly confidential. The proforma or record on which the information is to be put will not have your name on it but it will be linked with your clinic or hospital identification number. The custodian of this confidential linking information will be a physician at your clinic. Researchers other than your regular doctor will not have access to this linking information.

No personal information leading to identification of any individual taking part in the study will be included in any scientific publication.

- What will happen to the results of the research study?

Results will be published and fed back to every participant centre.

- Who is organising and funding the research?

The research team includes members of the Royal Free and University College Medical School staff and also personnel from the Clinical Trials Unit of the Medical Research Council and from the London School of Hygiene and Tropical Medicine. The principal researcher is Dr. Alejandro Arenas-Pinto who is a PhD student and this case-control study will form part of his PhD.

- Who has reviewed the study?

The London Multi-Centre Research Ethics Committee has approved this research.

- Contact for further information:

Dr.
Camden Primary Care Trust.
The Mortimer Market Centre.
Mortimer Market
off Capper Street
London WC1E 6AU

Telephone number:
E-mail:

Appendix 2

Papers and abstracts published

